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Herbicidal action mechanism of chlorsulfuron in the inhibition of sucrose translocation in a susceptible species, *Brassica napus* L. cv Westar.

by

Songmun Kim ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of
the requirements for the degree of Doctor of Philosophy

in

Weed Science

Department of Plant Science

Edmonton, Alberta

Fall, 1995

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Supervisor

John Hoddinott

External examiner

Date 2 Oct 95

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I-
I took the one less traveled by,
And that has made all the difference.

- Robert Frost

For Dr. William H. Vanden Born
and Dr. Dae-Sung Han,
who have always encouraged me
to do what I thought was right.

ABSTRACT

The herbicidal action mechanism of chlorsulfuron in inhibition of assimilate translocation was examined using canola (*Brassica napus* L. cv Westar) seedlings. Chlorsulfuron-treated leaves exported less exogenous ^{14}C-sucrose than control leaves (10% vs 23% of total recovered radioactivity). In addition, chlorsulfuron-treated excised leaves exuded about one fourth of the amount of sucrose exported by corresponding control leaves, suggesting that a reduction in assimilate translocation can be accounted for by a herbicide effect in the treated leaves.

Although chlorsulfuron-treated leaves exported less assimilate, the treated leaves showed no reduction in CO_2 fixation or carbohydrate production during the first 24 hours. However, during that time noticeable changes occurred in nitrogen metabolism, *i.e.*, increases in free amino acids (78%) and in nitrate reductase activity (two-fold). These changes in nitrogen metabolism, on a time scale, occurred later than the reduction in sucrose export. Activities of phosphoenol pyruvate carboxylase and sucrose phosphate synthase were not changed in treated leaves, suggesting that chlorsulfuron did not alter carbon flow away from sucrose to amino acids. Twenty-four hours after chlorsulfuron treatment, treated leaves had a higher respiration rate than control leaves, during an 8-hour experiment period. Thereafter, treated leaves had higher levels of carbohydrates such as hexoses and starch than control leaves. Chlorsulfuron-treated excised leaves exposed to high CO_2 concentration showed increased net carbon exchange rates and starch content (24%). However, such exposure did not overcome the reduction in sucrose transport.

When ^{14}C-sucrose was applied to a leaf adjacent to a chlorsulfuron-treated leaf, that leaf showed reduced export of ^{14}C-sucrose, suggesting a herbicide effect in plant parts other than the treated leaf. Chlorsulfuron (0.25 µg) applied directly to a sink leaf of a seedling with one source leaf and one sink leaf reduced ^{14}C-sucrose transport by 50%, presumably due to a reduction in sink strength (sink size and sink activity). The dry weight and cell numbers of treated

leaves were 40% and 82% lower, respectively, than those of control leaves. These results suggest that chlorsulfuron reduced assimilate translocation by reducing sink strength or sink demand for carbohydrates.

Supplementation with branched-chain amino acids or branched-chain amino acids and pantothenic acid prevented at least partially chlorsulfuron-induced growth inhibition. Chlorsulfuron-treated seedlings supplemented with branched-chain amino acids and pantothenic acid could prevent leaf rolling and chlorosis whereas the treated seedlings supplemented with branched-chain amino acids prevented only leaf rolling. These results suggest that a shortage of branched-chain amino acids and pantothenic acid is associated with the phytotoxicity in chlorsulfuron-treated plants. Canola plants treated with 2-ketobutyrate showed different changes in metabolites than plants treated with chlorsulfuron. The first leaves of 2-ketobutyrate-treated seedlings had similar concentrations of total sugar and free amino acids to those of control seedlings, indicating that 2-ketobutyrate did not induce the phytotoxicity found in chlorsulfuron-treated canola seedlings.

The overall results from this research show a model for the mechanism of action of chlorsulfuron in the reduction of assimilate translocation: a disruption of source-sink relations.

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1. INTRODUCTION

The action of herbicides originates with their ability to inhibit physiological and metabolic processes that are essential for plant growth. For initiation of herbicide action, applied herbicides must reach target sites through uptake and translocation processes. At the target site, the herbicide-target interaction inhibits a specific metabolic process in which the target enzyme or binding site is involved. The interaction also may affect many other metabolic and physiological processes as secondary and/or tertiary processes. At present, the interaction of many commercial herbicides with their targets is known. Although the herbicide-induced disturbances in many metabolic and physiological processes are known fragmentarily, the mechanisms connecting herbicide-target interaction with the herbicide-induced disturbances in a variety of metabolic and physiological processes are generally unknown (Moreland 1980).

Recently developed amino-acid-biosynthesis-inhibiting herbicides are exceptions. Several groups of herbicides inhibit enzymes in amino acid biosynthesis that are unique in plants (Duke 1990). Although the interactions between the enzymes and herbicides are known, it is not known how the primary interaction results in other disturbances in the metabolism and physiology of herbicide-treated plants. An understanding of the missing links connecting the enzyme-herbicide interaction and the cascade of metabolic disturbances is essential to know how the herbicides kill plants.

It has long been known that chlorsulfuron[1], one of the sulfonylurea herbicides, inhibits acetolactate synthase in branched-chain amino acid synthesis (Chaleff and Mauvais 1984). Although no researchers have shown evidence that chlorsulfuron depletes branched-chain amino acids in treated plants, many metabolic and physiological changes could be related to a shortage of branched-chain amino acids. This, however, does not explain observed changes in carbohydrate status in treated plants. Several researchers have shown that chlorsulfuron decreased assimilate transport out of treated leaves of susceptible plants such as *Thlaspi arvense* (Bestman *et al.* 1990a, Vanden Born *et al.* 1988) and *Arabidopsis thaliana* (Hall and Devine 1993). Chlorsulfuron-treated leaves of *Thlaspi arvense* did not show a change in CO_2 fixation but they accumulated carbohydrates. These results led Bestman *et al.* (1990a) to conclude that the limited translocation of assimilates was due to a herbicide effect on phloem loading in chlorsulfuron-treated plants. Although some researchers (Bestman *et al.* 1990a, Lowther 1990) suggested putative mechanisms connecting the site of action of chlorsulfuron and the phloem loading of assimilates, the mechanism(s) have not been fully elucidated.

---

[1] 2-chloro-N-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino-carbonyl]benzenesulfonamide (chemical name); DPX-W4189 (code name); Glean®.

The overall objective of this study was to develop an understanding of the mechanism of action of chlorsulfuron on assimilate translocation in canola, a susceptible species (*Brassica napus* L. cv Westar). The objective was attained by examining the following questions: (1) What is the effect of chlorsulfuron on growth of canola seedlings? (2) Why is growth of chlorsulfuron-treated canola seedlings reduced? Is it due to a shortage of branched-chain amino acids and pantothenic acid, or an accumulation of toxic intermediates? (3) Does chlorsulfuron affect assimilate transport? (4) Is the herbicide effect on assimilate transport due to a disruption of source-sink relations? (5) Does chlorsulfuron affect carbohydrate metabolism, carbohydrate production and allocation, in canola? (6) Is sink strength reduced by chlorsulfuron? (7) Does additional carbon dioxide overcome the herbicide-induced inhibition of assimilate transport? The same questions also were examined using herbicides (ethametsulfuron[2] and imazamethabenz-methyl[3]) with the same site of action as chlorsulfuron, a herbicide (glyphosate[4]) with a site of action in a different amino-acid-biosynthesis process, and a herbicide (clopyralid[5]) with a site of action in auxin-binding enzymes. The questions were tested by (1) supplementing canola seedlings with metabolites that have been suggested to inhibit or prevent growth of chlorsulfuron-treated plants, (2) applying ^{14}C-

---

[2] Methyl-2-[(4-ethoxy-6-methyl-1,3,5-triazin-2-yl)amino-carbonyl)benzenesulfonamide; DPX-A7881; Mustar ®.

[3] (±)-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-4 (and 5)-methylbenzoic acid (3:2); AC222,293; Assert®.

[4] N-(phosphomethyl)glycine; Roundup®.

[5] 3,6-dichloro-2-pyridinecarboxylic acid; Lontrel®.

sucrose to chlorsulfuron-treated seedlings and subsequently determining its translocation, (3) collecting sugar exudate from chlorsulfuron-treated excised leaves, (4) determining rates of many metabolic and physiological processes that are related to carbon fixation, (5) measuring carbohydrate production and allocation in chlorsulfuron-treated leaves, (6) reducing the sink strength of surgically modified seedlings and subsequently determining the pattern of sucrose translocation in the seedlings, or (7) exposing chlorsulfuron-treated excised leaves to high CO_2 concentrations and subsequently determining sugar exudation out of the leaves. Canola was selected as the experimental plant on the basis of high sensitivity to chlorsulfuron and rapid growth.

Canola seedlings treated with ethametsulfuron, imazamethabenz-methyl, and clopyralid did not show the metabolic and physiological changes found in seedlings treated with chlorsulfuron or glyphosate. Chlorsulfuron- and glyphosate-treated canola seedlings showed similar effects on assimilate translocation, however, carbon status in those seedlings was different. The metabolic and physiological changes including sucrose translocation in glyphosate-treated canola seedlings generally agreed with those in many other plants. Actions of herbicides other than chlorsulfuron in canola seedlings, therefore, were used as references. The results from all these experiments also raised the question: How do the consequences of herbicide-imposed stress on assimilate transport differ from those of other stresses such as, for example, chilling temperature stress? Although chilling-temperature-treated canola seedlings showed some metabolic and physiological changes similar to those in chlorsulfuron-treated seedlings, this report is focused primarily on the effects of chlorsulfuron.

2. LITERATURE REVIEW

A target of chlorsulfuron is the enzyme acetolactate synthase (ALS) in the branched-chain amino acid (BCAA) synthesis process, occurring in plastids. After chlorsulfuron-ALS interaction, many physiological and metabolic disruptions occur in plants. However, it is not known how chlorsulfuron-ALS interaction affects those physiological and metabolic processes and how this interaction leads subsequently to plant death. Similar to other physiological and metabolic processes which are reported to be changed by chlorsulfuron, a reduction of assimilate translocation is not well understood, even though the first report on it was published seven years ago (Bestman 1988). The understanding of herbicidal action and of assimilate translocation is essential to know the effect of chlorsulfuron on assimilate translocation.

A topic of the first part of this review is herbicide action[6]. Especially the interaction of ALS with chlorsulfuron (site of action) and the metabolic and physiological changes that follow (mechanism of action) have been reviewed. In the last few years, extensive studies on interactions of ALS-enzyme inhibitors have provided information about reaction mechanisms of ALS. Studies on structure-activity relationships between several groups of inhibitors and ALS as well as on structural differences of ALS between susceptible and resistant biotypes have helped to understand the site of action of chlorsulfuron. However, only a few studies have shown how plants responded after the interaction of the ALS and chlorsulfuron and how the site and/or mechanism of action of chlorsulfuron lead to physiological and metabolic disruptions in plants.

In the second part of this review, production, allocation, and translocation of sucrose in fully expanded leaves of plants are reviewed in order to understand how sucrose translocation can be regulated. In addition, fates of translocated assimilates are reviewed because disturbances of this complex process in actively growing plant parts can affect the overall mechanisms of production, allocation, and translocation of assimilates. In the last decade, these have been studied intensively and the information has expanded dramatically. Because of the large amount of information available only a general review is presented.

---

[6] Many different terms have been used to describe the action of herbicides. In this thesis, the "site of action" specifically means the interaction of herbicide molecule with the target enzyme. The "mechanism of action" refers to the whole complex of herbicide-plant interferences that result from the herbicide-target enzyme interaction.

In conjunction with assimilate translocation, an inductive linkage of the site of action of herbicide with mechanisms of action on assimilate translocation will help to understand the subject of this study - a reduction of assimilate translocation in plants.

2.1. Herbicide actions of chlorsulfuron

2.1.1. Site of action

Biosynthesis of BCAA (leucine, valine, and isoleucine) occurs in the plastids of higher plant cells. This process has been of particular interest to many weed scientists as well as herbicide developers because it is not only unique in plants but also contains several enzymes as possible herbicide targets. As herbicide targets, three enzymes in the BCAA synthesis process were studied: acetolactate synthase (ALS; also referred as acetohydroxyacid synthase, AHAS), acetolactate reductoisomerase (Schulz *et al.* 1988), and isopropyl malate dehydrogenase (Wittenbach *et al.* 1994). Through this pathway, plants produce the BCAA and pantothenic acid. The biosynthetic process of BCAA, including enzymes that catalyze each step, is illustrated in Fig. 1.

Many researchers have shown that the target of chlorsulfuron, a herbicide of the sulfonylurea family, is ALS. In addition to the sulfonylureas[7] (LaRossa and Schloss 1984, Ray

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[7] The compounds are developed by E. I. Du Pont de Nemours & Co. Structures and potency of the compounds are in US Patent 4 169 719 and 4 127 405

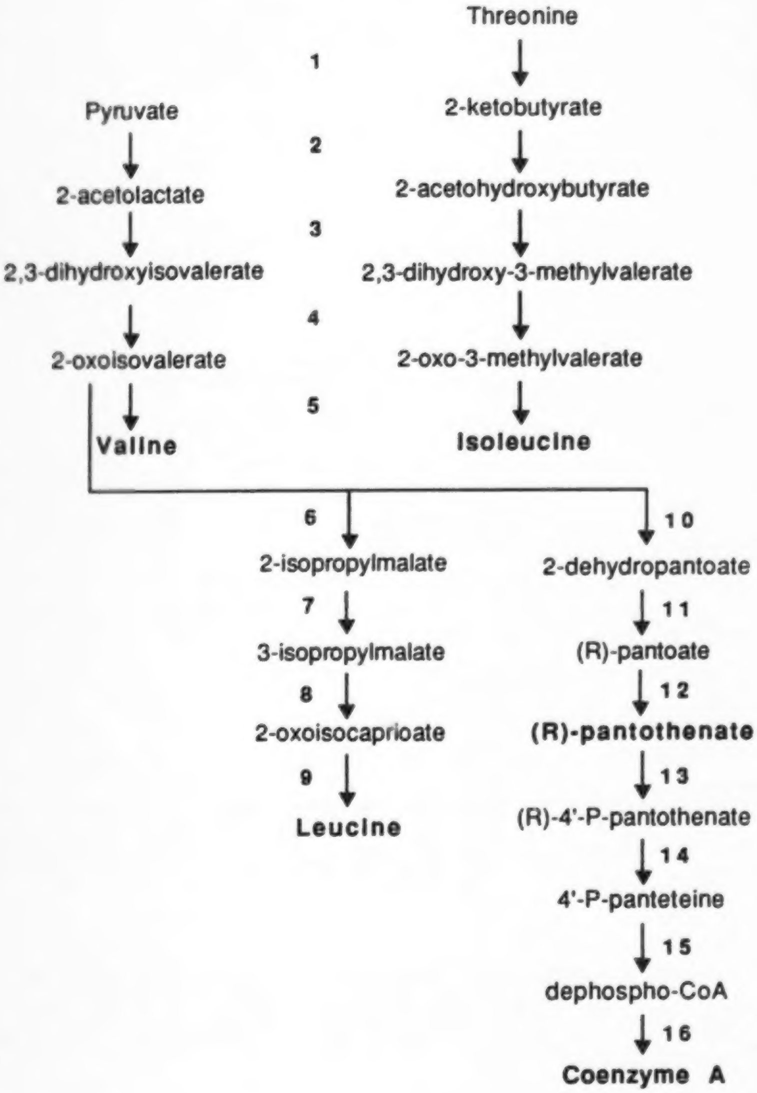
Threonine
1
2-ketobutyrate
Pyruvate
2
2-acetolactate
2-acetohydroxybutyrate
3
2,3-dihydroxyisovalerate
2,3-dihydroxy-3-methylvalerate
4
2-oxoisovalerate
2-oxo-3-methylvalerate
5
Valine
Isoleucine
6
10
2-isopropylmalate
2-dehydropantoate
7
11
3-isopropylmalate
(R)-pantoate
8
12
2-oxoisocaprioate
(R)-pantothenate
9
13
Leucine
(R)-4'-P-pantothenate
14
4'-P-panteteine
15
dephospho-CoA
16
Coenzyme A

Figure 1. Biosynthesis of branched-chain amino acids and pantothenic acid. Numbered steps are catalyzed by enzymes: 1, threonine deaminase; 2, acetolactate synthase; 3, acetolactate reductoisomerase; 4, dihydroxyacid dehydratase; 5, amino transferase; 6, isopropylmalate synthase; 7, isopropylmalate isomerase; 8, isopropylmalate dehydrogenase; 9, aminotransferase; 10, 3-methyl-2-oxobutanoate hydroxymethyl transferase; 11, 2-dehydropantoate reductase; 12, pantothenate-β-alanine ligase; 13, pantothenate kinase; 14, *P*-pantothenate cysteine ligase; 15, pantetheine-*P*-adenylyl-transferase; 16, dephospho-CoA-kinase. Three enzymes are targets of herbicides and experimental chemicals: 2, sulfonylureas, imidazolinones, triazolopyrimidine sulfonanilides, and pyrimidinyl oxobenzoic acids; 3, 2-methyl-phosphinoyl-2-hydroxyacetic acid (HOE-704) and *N*-isopropyl oxalylhydroxamate (IpOHA); 8, *O*-isobutenyl oxalylhydroxamate (*O*-IbOHA).

1984), three other families of highly active herbicides, namely, imidazolinones[8] (Shaner *et al.* 1984, Muhitch *et al.* 1987, Hawkes 1989, Stidham and Shaner 1990), triazolopyrimidine sulfonanilides[9] (Gerwick *et al.* 1990, Kleschik *et al.* 1990), and pyrimidinyl oxobenzoic acids[10] (Shimizu *et al.* 1994, Takahashi *et al.* 1991, Hanai *et al.* 1993) are also potent inhibitors of ALS. Although these inhibitors are structurally different, herbicide actions occur at the same target site.

ALS catalyzes the condensation of two molecules of pyruvate to form 2-acetolactate and CO_2, as the first common step of leucine and valine biosynthesis, or one molecule of pyruvate and one of 2-ketobutyrate to form 2-acetohydroxybutyrate and CO_2, as the common step of isoleucine biosynthesis (Fig. 1). Thiamine pyrophosphate and a metal ion, Mn^{2+} or Mg^{3+}, are required (Schloss *et al.* 1988, Singh and Schmitt 1989, Durner and Böger 1990).

ALS is a nuclear encoded but plastid-located enzyme and is found in bacteria, fungi, algae, and plants (Miflin 1974, Schloss *et al.* 1985). Genes for ALS have been characterized in bacteria, fungi, and plants. Bacterial ALS from *E. coli* and *Salmonella typhimurium* and plant ALS from *Arabidopsis thaliana*, *Nicotiana tabacum*, and *Brassica napus* (Haughn *et al.* 1988, Relton *et al.* 1986, Bekkaoui *et al.* 1993) have been studied in detail. Three genes coding for isozymes were cloned (I, II, and III). Isozymes of ALS are reported to have different specificities for substrates, pyruvate and 2-ketobutyrate. In the bacterium *S. typhimurium*, isozyme II is catalytically competent in the reaction, while isozyme I utilizes 2-ketobutyrate inefficiently (De Felice 1982). Isozyme II is a tetramer that is composed of two large subunits (60 kDa) and two small subunits (9.7 kDa). In plants, the structure of ALS appears more diverse and potentially more complex. Molecular masses of the non-denatured ALS enzyme ranges from 55 kDa to 440 kDa in different species. Variations were observed also within the same species and when different tissues were used (Durner and Böger 1988).

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[8] The compounds are developed by American Cyanamid Co. Structures and potency of the compounds are in US Patent 4 544 754 and 4 798 619.

[9] Derivatives of 1,2,4-triazolo[1,5-a]-pyrimidines. The compounds are developed by Dow Elanco. Structures and potency of the compounds are in Pestic. Sci. 1990. 29:341-355

[10] Derivatives of 2-(4',6'-di-substituted pyrimidine-2'-yl)oxy- or thio-benzoic acid. The compounds are developed by Kumiai Chemical Industry Co., Ltd. Structures and potency of the compounds are in European Patent 0 223 406.

Although ALS is the target of chlorsulfuron, exact binding sites on the enzyme are not known. The information about the site of action of chlorsulfuron comes from three different studies: (1) kinetics of ALS *in vitro*, (2) genetics of ALS, and (3) structure-activity relationships between ALS and chlorsulfuron.

Studies on the kinetics of ALS in bacteria and higher plants show how chlorsulfuron interacts with ALS. Some studies provided strong evidence that the binding of chlorsulfuron to the enzyme is reversible and slow (Bastide and Orteta 1995, Schloss 1993). However, these conclusions are controversial, because chlorsulfuron could physically dissociate from the ALS enzyme without any corresponding recovery of activity (Durner *et al.* 1991). Hawkes (1993) proposed that the slow process might not correspond to the formation of a tight complex, but rather to an irreversible change in conformation of the enzyme with little or no catalytic activity. More work is needed to understand clearly under what conditions ALS activity is lost, and the process that is involved. Schloss *et al.* (1988) found that two water-soluble ubiquinone homologues (Q_0 and Q_1) were potent inhibitors of bacterial isozymes of ALS. And chlorsulfuron caused time-dependent inhibition with at least one of the isozymes, suggesting that the herbicide-specific site in ALS is an evolutionary vestige of the quinone-binding site of pyruvate oxidase.

In genetic analysis of sulfonylurea-resistant plants, Chaleff and Mauvais (1984) showed that the resistance is linked to a single semi-dominant nuclear gene mutation. Based on a study of herbicide-resistant mutants of *Nicotiana thaliana*, Lee *et al.* (1988) reported that one mutant, C3, had a single proline-glutamine replacement at amino acid 196 in one ALS gene, while the other mutant, S4-Hra, has two amino acid changes in the other ALS gene, a proline-alanine substitution at 196 and a tryptophan-leucine substitution at 573. Similar results were obtained in mutant *Arabidopsis* (Haughn *et al.* 1988), *Brassica* (Bekkaoui *et al.* 1993) and *Zea* (Fang *et al.* 1992). These amino acid substitutions may be responsible for herbicide resistance.

As ALS is regulated by Leu and Val, it contains feedback regulatory site(s) for those amino acids. However, it is not clear whether the chlorsulfuron-binding site overlaps or is distinct from the feedback regulation site (Hawkes 1993). Subramanian *et al.* (1991) had reported that the herbicide binding and feedback regulatory sites in ALS overlapped. The evidence was presence (overlapping) of cross-resistance of mutant enzymes. At present, structure-activity relationships between chlorsulfuron and ALS are likely to be the only available approach to elucidate the herbicide-binding site. In such a study of sulfonylurea herbicides, Andrea *et al.* (1992) proposed a putative herbicide-binding site in ALS. However, as authors commented, the exact binding site of chlorsulfuron would not be obtained until a crystal structure of homogeneous ALS became available.

In conclusion, several lines of evidence support the target of chlorsulfuron is the ALS in the BCAA biosynthesis process. However, the exact binding site of chlorsulfuron on ALS is not known because of lack of information on the quaternary structure of ALS.

2.1.2. Mechanism of action

After chlorsulfuron binds to the ALS in plastids, many physiological and metabolic changes occur as secondary or tertiary components of the mechanism of action of chlorsulfuron (Table 1). These changes are likely to be linked to a growth inhibition that eventually leads to plant death. Although these changes have been reported to occur as a result of inhibition of the site of action of chlorsulfuron, it is not known how the inhibition of the site of action of chlorsulfuron leads to those physiological and metabolic changes.

In many plants, chlorsulfuron is known to stop cell division in meristematic tissues such as root tips (Ray 1980, Ray 1984, Rost 1984, Rost and Reynold 1985, Robbins and Rost 1987). Exposure to 1 mg ml^{-1} chlorsulfuron caused a large drop in mitotic indices of field bean and pea roots (Ray 1980, Ray 1982a). In pea roots, Rost (1984) found that chlorsulfuron blocked cell-cycle progression in both the G1 (pre-DNA synthesis) and G2 (pre-mitotic) phases, without interfering directly with mitosis and DNA synthesis and suggested that a primary block for cell-cyle progression was in G2 and the G1 block was a secondary and possibly weaker block. The

Table 1. Physiological and metabolic changes in chlorsulfuron-treated plants.

Physiological and metabolic change	Effect*	Species	Reference
DNA synthesis	-	Soybean	Ray (1980)
	no/-	*Phaseolus vulgaris*	De Villiers *et al.* (1980)
	no	Soybean	Ray (1982a)
	-	*Pisum sativum*	Ray (1982b)
	-	Soybean	Ray (1984)
	-	*Pisum sativum*	Rost (1984)
RNA synthesis	no/-	*Phaseolus vulgaris*	De Villiers *et al.* (1980)
	-	*Pisum sativum*	Ray (1982b)
	-	Soybean	Hatzios and Howe (1982)
	-	*Pisum sativum*	Rost (1984)
Protein synthesis	no	Soybean	Ray (1982a)
	no	*Pisum sativum*	Ray (1982b)
	no	*Pisum sativum*	Rost (1984)
	no	*Pisum sativum*	Clayton and Reynolds (1991)
	+/-	*Phaseolus vulgaris*	De Villiers *et al.* (1980)
	+/-	Soybean	Hatzios and Howe (1982)
Lipid synthesis	no/-	*Phaseolus vulgaris*	De Villiers *et al.* (1980)
	-	Soybean	Hatzios and Howe (1982)
	-	Cotton	Trufanova (1990a, b)
Cell wall synthesis	no	*Arabidopsis thaliana*	Heim *et al.* (1990)
Plant growth regulator			
Ethylene synthesis	+	Soybean	Suttle and Schreiner (1982)
	+	Sunflower	Suttle *et al.* (1983)
	+	Velvetleaf	Hageman and Behrens (1984)
Anthocyanins production	+	Soybean	Suttle and Schreiner (1982)
Polyamines			
Spermidine	-	Maize	Giardina and Carosi (1990)
	no	Pea	DiTomaso (1988)
Putrescine	+	Pea	DiTomaso (1988)
Cadaverine	+	Pea	DiTomaso (1988)
Amino acid content			
Total amino acid	+	Field pennycress	Bestman *et al.* (1990a)
	+	Maize	Royuela *et al.* (1991)
	no	Soybean	Scheel and Casida (1985)
BCAA	-	*Lemna minor*	Rhodes *et al.* (1987)
	-	Maize	Royuela *et al.* (1991)
	-	Soybean	Scheel and Casida (1985)

Physiological and metabolic change	Effect*	Species	Reference
Toxic metabolites			
2-oxobutyrate			
2-aminobutyrate	+	*Lemna minor*	Rhodes *et al.* (1987)
	no	Maize	Shaner and Singh (1993)
Sugar content	+	Field pennycress	Bestman *et al.* (1990a)
	+	Field Pennycress	Bestman *et al.* (1990b)
PAL activity	+	Soybean	Suttle and Schreiner (1982)
Ion uptake and transport			
Ca^{2+} absorption and translocation	-	maize	Crowley and Prendeville (1985)
K$^+$ uptake			
H$^+$ extrusion	-	maize	De Agazio and Giardina (1987)
ATPase activity	no	*Phaseolus vulgaris*	De Villiers *et al.* (1980)
	no	*Arabidopsis thaliana*	Hall and Devine (1993)
Photosynthesis			
Oxygen evolution	no	*Phaseolus vulgaris*	De Villiers *et al.* (1980)
Carbon fixation	no	Field bean	Ray (1980)
	+/-	*Phaseolus vulgaris*	DeVilliers *et al.* (1980)
	no	Soybean	Ray (1982a)
	no	*Pisum sativum*	Ray (1982b)
	no	Soybean	Hatzios and Howe (1982)
	no/-	*Phaseolus vulgaris*	DeVilliers *et al.* (1985)
	no	Wheat	Cink *et al.* (1985)
Respiration	no	*Pisum sativum*	Ray (1982a)
Assimilate transport	-	Field pennycress	Vanden Born *et al.* (1988)
	-	Tartary buckwheat	Devine *et al.* (1990)
	-	*Arabidopsis thaliana*	Hall and Devine (1993)
Cell division	-	Field bean	Ray (1980)
	-	Pea	Ray (1982b)
	-	*Pisum sativum*	Rost (1984)
Cell elongation	no	Pea, cucumber, lettuce	Ray (1980)
Plant growth	-	Many species	Many researchers

* "-" designates negative effects such as inhibition and reduction, while "+" represents positive effects such as activation and stimulation. "+/-" designates that herbicide effects are positive at lower concentrations while they are negative at higher concentrations. "no/-" designates that herbicide effects are not apparent at lower concentrations while they are negative at higher concentrations.

inhibition of cell-cycle progression was prevented by supplementation with Ile and Val (Robbins and Rost 1987), implying that a cessation in cell division was a secondary mechanism of action of chlorsulfuron and the BCAA were involved in a cell-cycle control mechanism. Transitions from G1 to S and from G2 to M are known to be arrested by G2 factors, such as trigonellines[11] and 5-methylnicotinic acid (Evans *et al.* 1979). Depletion of nutrients such as carbohydrate also arrests the cell cycle in G2 and G1 (Gould *et al.* 1981). In addition, certain specific proteins must be coded and synthesized and certain initial amounts of RNA are required for cell-cycle transitions (Webster and van't Hof 1970). Regarding the cell division inhibition in chlorsulfuron-treated plants, no putative mechanism has been published. Further studies on cell-division-related biochemical and biophysical events will help to understand the mechanism connecting the site of action of chlorsulfuron with a concomitant effect on cell division.

Chlorsulfuron increased levels of polyamines such as putrescine and cadaverine in pea, without affecting levels of spermidine and spermine (DiTomaso 1988). However, Giardina and Carosi (1990) reported that chlorsulfuron decreased spermidine only in meristematic tissues of maize, without affecting other polyamines such as putrescine and spermine, suggesting that a chlorsulfuron-induced change in spermidine content could be responsible for cell-cycle-progression inhibitions in stages G1 and G2. Since polyamines regulate cell-cycle progressions (Evans and Malmberg 1989), suggestions by both groups of researchers are reasonable. In chlorsulfuron-treated plants, it is not known how chlorsulfuron disrupts polyamine balances and whether a disruption in polyamine balances leads to an inhibition in cell division.

DNA synthesis was inhibited by chlorsulfuron in some plants. In soybean, for example, chlorsulfuron inhibited the incorporation of ^{3}H-thymidine into DNA (Ray 1980, Rost 1984, de Villiers *et al.* 1980). However, in isolated soybean hypocotyl tissue, chlorsulfuron did not have an effect on DNA polymerase and thymidine kinase, enzymes involved in DNA synthesis (Ray 1982a).

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[11] 3-carboxy-1-methylpyridinium hydroxide; $C_7H_7NO_2$

Chlorsulfuron inhibited RNA synthesis processes in several plants. In isolated cells of *Phaseolus vulgaris*, de Villiers *et al.* (1980) reported a 67% reduction in RNA synthesis. However, this occurred only at a relatively high concentration of chlorsulfuron (0.17 mg ml^{-1}). Rost (1984) showed that chlorsulfuron-treated pea roots incorporated less ^3H-uridine than control roots and suggested that chlorsulfuron might inhibit cell-cycle-specific RNAs. The cell cycle was blocked primarily at G2 and secondarily at G1. At present, it is not clear whether the inhibition of cell-cycle-specific RNAs is associated with a shortage of BCAA.

Chlorsulfuron inhibited protein synthesis, but only at high concentrations. de Villiers *et al.* (1980) reported that chlorsulfuron reduced protein synthesis at higher than 36 μg ml^{-1} by more than half. Similar results were obtained with isolated soybean leaf cells (Hatzios and Howe 1982). Because of the mechanism of action of chlorsulfuron, a shortage of specific amino acids is expected, as a result of which the rate of protein synthesis could be reduced and protein profiles in plants changed. However, protein profiles in root tips of chlorsulfuron-treated *Pisum sativum* were not changed (Clayton and Reynolds 1991). In fact, protein synthesis was not affected at a concentration of chlorsulfuron that could affect cell division (Ray 1982a, Clayton and Reynolds 1991), implying that cell division is more sensitive than protein synthesis to chlorsulfuron. At present, it is not known whether an inhibition of *de novo* protein synthesis is associated with a shortage of BCAA or any other factors that are involved in the protein synthesis process.

The size of the free amino acid pool was increased in chlorsulfuron-treated plants such as *Lemna minor* (Rhodes *et al.* 1987) and *Thlaspi arvense* (Bestman *et al.* 1990a). In *Lemna minor*, for example, chlorsulfuron treatment increased levels of several amino acids such as alanine, proline, ornithine, methionine and 2-aminobutyrate, while it decreased levels of serine, methionine, and BCAA (Rhodes *et al.* 1987). An impaired utilization of amino acids in protein synthesis and an increase in proteolysis accounted for changes in amino acids (Singh and Shaner 1995). Aminooxyacetate, a known inhibitor of transaminase and glycine decarboxylase, promoted an extensive accumulation of several amino acids including BCAA (Brunk and Rhodes 1988). However, chlorsulfuron did not inhibit aminooxyacetate-induced BCAA accumulation, suggesting that the accumulation of BCAA in chlorsulfuron-treated *Lemna* is due to proteolysis. In addition to proteolysis, synthesis of free amino acids could possibly increase if chlorsulfuron increased productions of amino acid precursors and activated amino acid biosynthesis processes.

An interaction of chlorsulfuron with ALS leads to an accumulation of 2-ketobutyrate and 2-aminobutyrate in plants (Rhodes *et al.* 1987). These compounds are toxic to microorganisms and plants (LaRossa and Van Dyk 1987, Schloss 1989). Danchin *et al.* (1984) suggested that the final target of 2-ketobutyrate was a component of complex PEP-dependent carbohydrate phosphotransferase which can result in a decline of concentrations of glycolytic intermediates either upstream (G6P, F6P, F16BP) or downstream (acetyl CoA) from PEP. In addition, 2-ketobutyrate has been known to inhibit γ-glutamyl cysteine synthase in glutathione biosynthesis (Sekura and Meister 1977) and methionyl-tRNA synthase (Hahn and Brown 1967). At higher concentrations (4-6 mM), this compound is also known to inhibit cell division in *Allium* root tips (Lanzagorta *et al.* 1988) and to acidify the cytoplasm which subsequently stops cell division in *Hordeum* root tips (Reid *et al.* 1985). These results suggest that chlorsulfuron-induced phytotoxicity could be caused by an accumulation of 2-ketobutyrate or 2-aminobutyrate. However, 2-ketobutyrate is a product of threonine dehydratase (or deaminase) which is regulated by Ile. If an accumulation of 2-ketobutyrate is an important factor in chlorsulfuron-induced phytotoxicity, chlorsulfuron-treated plants should show a prevention of the phytotoxicity by supplementation with Ile. However, chlorsulfuron-induced phytotoxicities were not prevented by supplementation with Ile (Shaner and Singh 1992, Shaner and Singh 1993), suggesting that phytotoxicities of ALS-inhibiting herbicides are not due to an accumulation of 2-ketobutyrate or 2-aminobutyrate.

Ethylene synthesis in chlorsulfuron-treated plants was higher than that in untreated plants. In velvetleaf, for example, chlorsulfuron increased ethylene production and cellulase activity (Hageman and Behrens 1984). Similar results on ethylene production were observed in chlorsulfuron-treated soybean hypocotyls (Suttle and Schreiner 1982) and sunflower seedlings (Suttle *et al.* 1983). Although chlorsulfuron increased ethylene synthesis, it did not affect the synthesis of other plant hormones. IAA-induced elongation of subapical etiolated pea stems, cytokinin-induced cell expansion of cucumber cotyledons, and gibberellic-acid-induced elongation of lettuce hypocotyls were not affected by chlorsulfuron (Ray 1982b), implying that chlorsulfuron did not inhibit cell elongation processes.

Chlorsulfuron affected uptake and transport of ions through membranes. In maize seedlings, chlorsulfuron caused an alteration of plasma membrane function with respect to fusicoccin (FC)-stimulated K^+ uptake and H^+ extrusion (de Agazio and Giardina 1987). The effect of chlorsulfuron was not prevented by supplementation with BCAA. The authors suggested an alternative site of action of chlorsulfuron in maize plants.

Photosynthetic processes, including oxygen evolution and carbon fixation, were affected at relatively higher concentrations of chlorsulfuron. In *Phaseolus vulgaris*, chlorsulfuron reduced photosynthetic processes at higher than 0.036 mg ml^{-1} (de Villiers *et al.* 1980). Other researchers also reported similar results (Cink *et al.* 1985, Hatzios and Howe 1982, Ray 1980, Ray 1982b). In *Thlaspi arvense*, for example, chlorsulfuron did not affect photosynthetic processes at a concentration of 0.1 mg ml^{-1}(Bestman *et al.* 1990a).

Chlorsulfuron reduced assimilate translocation in plants such as Tartary buckwheat (Devine *et al.* 1990), *Thlaspi arvense* (Vanden Born *et al.* 1988, Bestman *et al.* 1990a), and *Arabidopsis thaliana* (Hall and Devine 1993). Chlorsulfuron at 0.1 mg ml^{-1} reduced the translocation of sucrose in such plants without affecting photosynthesis. The chlorsulfuron effect on assimilate translocation was partially prevented by supplementation with BCAA (Vanden Born *et al.* 1988, Devine *et al.* 1990), suggesting that a reduction in assimilate transport is related to a shortage of BCAA. Malfunctions of membranes (Vanden Born *et al.* 1988) or proteins that are involved in phloem loading of assimilates (Bestman *et al.* 1990a, Lowther 1990) have been suggested as a putative mechanism for reduced assimilate transport in chlorsulfuron-treated plants. Although chlorsulfuron-treated *Arabidopsis thaliana* exported less assimilate than untreated plants, chlorsulfuron did not inhibit activities of the plasma membrane H^+-ATPase (Hall and Devine 1993) that have an important role in the phloem loading of assimilates (Michelet and

Boutry 1995). The fact that chlorsulfuron did not affect a function of the plasma membrane H^+-ATPase important in the phloem loading of assimilates raises a question on the putative mechanism for reduced assimilate transport. In plants, source leaf ability for carbohydrate production and allocation is interrelated, for assimilate transport, with sink ability for carbohydrate utilization. Therefore, a reduction of assimilate transport in plants might reflect a disruption of interrelations between source and sinks. Chlorsulfuron-treated plants that showed a reduction in assimilate transport had higher levels of soluble carbohydrates. Thirty hours after treatment, chlorsulfuron-treated leaves of *Thlaspi arvense* had higher amounts of total sugars and starch than control leaves (Bestman *et al.* 1990a, Lowther 1990). The reduction in assimilate transport could account for an accumulation of carbohydrates.

Chlorsulfuron reduced plant growth in many susceptible plants such as pea (Giardina *et al.* 1987), velvetleaf (Hageman and Behrens 1984), Canada thistle (Donald 1984), *Thlaspi arvense* (Vanden Born *et al.* 1988), Tartary buckwheat (Devine *et al.* 1990), and *Arabidopsis thaliana* (Hall and Devine 1993). The growth retardation of chlorsulfuron-treated plants is suggested to be caused by a depletion of BCAA and pantothenic acid, an accumulation of toxic metabolites, and/or a reduction of assimilate translocation (Ray 1984, LaRossa *et al.* 1987, Shaner and Singh 1992, Shaner and Singh 1993, Vanden Born *et al.* 1988). Although these putative mechanisms are related to a site of action of chlorsulfuron, it is not known how a result of the site of action, a shortage of BCAA, is linked to other putative mechanisms and how the linking mechanism leads the growth retardation of chlorsulfuron-treated plants.

In conclusion, several lines of evidence show that ALS is a target of chlorsulfuron and that the interaction of chlorsulfuron with ALS changes many metabolic and physiological processes. However, a clear mechanism that can connect the site of action of chlorsulfuron with those metabolic and physiological processes has not emerged. Understanding the missing links between the site of action of chlorsulfuron and the following cascade of metabolic and physiological changes will help to know how plants respond to chlorsulfuron and to other chemicals that inhibit both BCAA and other amino acid synthesis processes.

2.2. Production, allocation, and translocation of carbohydrates

Carbohydrates are translocated from regions of carbohydrate synthesis (source) to regions of utilization and/or storage (sink). The source is photosynthetically active organs such as mature leaves and the sinks are organs such as meristems and storage organs that may or may not be photosynthetically active. The source and sinks are interconnected for assimilate translocation, therefore limiting factors for carbohydrate production in the source affect metabolic and physiological functions in sinks, and *vice versa*. However, the source-sink relationships are not fully understood, though their mechanics and dynamics appear to determine the nature of plant growth (Gifford *et al.* 1984, Sonnewald and Willmitzer 1992). In this part of the review, the scope is focused to provide general ideas on production, allocation, and translocation of carbohydrates and interactions between source and sinks for carbohydrate translocation.

2.2.1. Production and allocation of carbohydrates

Dynamics of carbon dioxide fixation and carbohydrate production are very complex, requiring interactions of different organelles and metabolisms. The principal biochemical pathways of carbohydrate production in leaves of C_3 plants are given in Fig. 2. Recently several comprehensive reviews on sugar metabolism in plants have been published (Hawker *et al.* 1991, Kleczkowski 1994, Stitt and Quick 1989, Sonnewald *et al.* 1994).

CO_2 is fixed by ribulose-1,5-bisphosphate carboxylase in chloroplasts (Hawker *et al.* 1991). The first products of carbon fixation are triose phosphates (triose-P) such as 3-phosphoglycerate (3-PGA) and dihydroxyacetone phosphate (DHAP). These metabolites play pivotal roles in carbon metabolism: the regeneration of ribulose-1,6-bisphosphate and the utilization for starch and sucrose synthesis. Starch is a major storage carbohydrate in most plants while sucrose is a major translocating carbohydrate. Energy required for carbon fixation is generated through a series of enzyme reactions in photosystems I and II. Final products of the

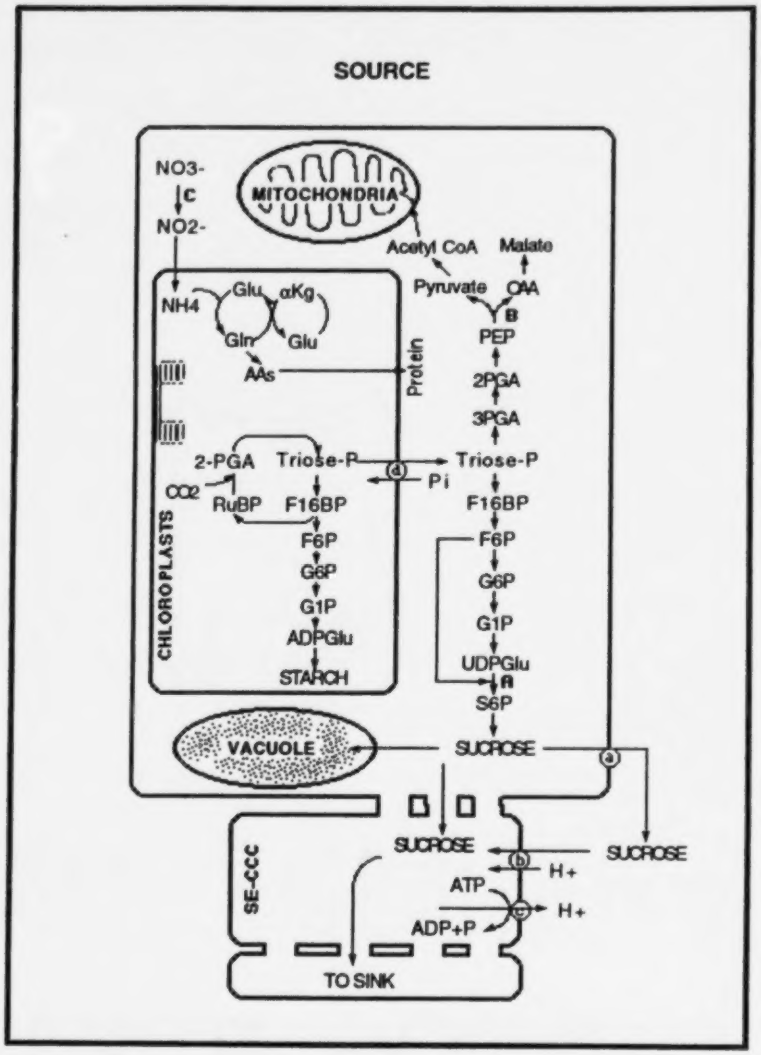
SOURCE
NO3-
C
NO2-
MITOCHONDRIA
Acetyl CoA
Malate
Pyruvate
OAA
B
PEP
2PGA
3PGA
NH4
Glu
αKg
Gln
Glu
AAs
Protein
2-PGA
Triose-P
CO2
RuBP
F16BP
F6P
G6P
G1P
ADPGlu
STARCH
CHLOROPLASTS
d
Pi
Triose-P
F16BP
F6P
G6P
G1P
UDPGlu
A
S6P
VACUOLE
SUCROSE
a
SUCROSE
b
SUCROSE
H+
ATP
c
H+
ADP+P
SE-CCC
TO SINK

Figure 2. Production, allocation, and phloem loading of carbohydrates in the source. Carbon dioxide is reductively assimilated in the chloroplasts. Triose phosphates transport out of the chloroplasts by the phosphate translocator (d) and, through a series of reactions, sucrose is synthesized. Triose phosphates are also utilized for respiration. Sucrose may be temporarily stored in the vacuole or it transports to sinks through phloem. Sucrose is transported into the phloem through plasmodesmata (symplastic phloem loading) and against a concentration gradient by the proton-sucrose symporter (b) (apoplastic phloem loading). The thermodynamic driving force for proton-sucrose symporter is a proton motive force (c). a, a putative sucrose transporter; **A**, sucrose phosphate synthase; **B**, phosphoenol pyruvate carboxylase ; **C**, nitrate reductase. Abbreviations; Glu, glutamate; Gln, glutamine; αKg, α–ketoglutarate; AAs, amino acids; 3-PGA, 3-phosphoglycerate; Triose-P, triose-phosphate; F16BP, fructose-1,6-phosphate; RuBP, ribulose bisphosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate; ADPGlu, ADP glucose; OAA, oxaloacetate; UDPGlu, UDP glucose; S6P, sucrose-6-phosphate; SE-CCC, sieve element-companion cell complex.

energy production reaction are ATP and NADPH (Krall and Edwards 1992). These are utilized for carbon fixation and other energy-requiring metabolic processes.

Triose-P formed during photosynthesis is converted to glucose-1-phosphate (Glu1P) by a series of enzyme reactions for starch synthesis in chloroplasts (Preiss 1988). Glu1P is converted to ADP-glucose, which is converted to amylose or amylopectin, by ADP-glucose pyrophosphorylase. Several different enzymes are involved in starch formation: ADP-glucose pyrophosphorylase, starch synthetase, and branching enzymes (Manners 1985, Okita 1992, Shewmaker and Stalker 1992). The major regulatory point for starch synthesis is ADP-glucose pyrophosphorylase that catalyzes the formation of ADP-glucose from Glu1P. In general, ADP-glucose pyrophosphorylase is inhibited by inorganic phosphate (Pi) and activated by 3-PGA.

Triose-P that is not used for starch synthesis is transported out of chloroplasts into cytosol in strict counter-exchange with Pi by phosphate translocators (Heldt *et al.* 1990, Willey 1991, Ohnishi *et al.* 1989). In the cytosol, triose-P is converted to fructose-1,6-phosphate (F1,6BP) which is utilized mainly for sucrose synthesis. The synthesis of sucrose is regulated at two steps (Huber and Huber 1992, Stitt and Quick 1989): a conversion of F1,6BP to fructose-6-phosphate (F6P) and a conversion of F6P and UDP-glucose to sucrose-6-phosphate (S6P). The first reaction is regulated by fructose-2,6-bisphosphate (F2,6BP) that inhibits cytosolic fructose-1,6-bisphosphatase (FBPase) and activates pyrophosphate: fructose-6-phosphotransferase (PFP). Because of its potent inhibition of FBPase, F2,6BP inhibits carbon flow toward sucrose synthesis and stimulates starch synthesis (Stitt 1990). The second reaction is mediated by S6P synthase (SPS) which is regulated by a ratio of G6P (activator) and Pi (inhibitor). The metabolic control of SPS activity provides a mechanism where accumulation of hexose phosphate occurs, which increases activity of FBPase and subsequently the rate of sucrose synthesis.

Newly synthesized carbon is allocated to plant components other than carbohydrates such as organic acids and amino acids. This mechanism also determines sucrose synthesis in C_3 plants (Champigny and Foyer 1992). Interactions between carbon and nitrogen affect sucrose synthesis, because carbon and nitrogen biochemical processes are related in several ways (Rufty *et al.* 1992, Stulen 1986). Firstly, carbon fixation and nitrogen assimilation require common energy sources such as ATP, NADPH, and reduced ferredoxin in chloroplasts. Secondly, the production of NADH in sucrose synthesis provides reducing equivalents for nitrate reduction. Thirdly, compartmentation of nitrate in the vacuole is influenced by carbohydrate status. Finally, carbon partitioning between sucrose and amino acids competes for carbon backbones and energy. Several researchers showed that nitrate functions as a signal metabolite for cytosolic protein kinase activation (Champigny and Foyer 1992, Quy *et al.* 1991, Quy and Champigny 1992), *i.e.*, activation of PEPcase and inactivation of SPS that resulted in an increase in free amino acids and a decrease in sucrose.

The synthesized sucrose is either translocated away or temporarily stored in vacuoles by a facilitated diffusion or H^+-sucrose antiporter (Stitt *et al.* 1987, Stitt *et al.* 1988).

2.2.2. Sucrose translocation

The generally accepted idea for carbohydrate translocation is an osmotic pressure flow hypothesis (Münch 1930). A high concentration of carbohydrate in the phloem of carbohydrate synthesis regions (sources) causes a water influx into the phloem (Smith and Milburn 1980). The generated osmotic potential in the phloem is responsible for long-distance carbohydrate transport through the positive hydrostatic pressure. In regions of carbohydrate utilization and storage (sinks), translocated carbohydrates are continuously metabolized and compartmentalized, generating a concentration gradient between source and sinks. In this system, carbohydrates load into the phloem (phloem loading) and unload out of the phloem (phloem unloading). In most plant species, sucrose is a major transporting sugar. However, in some species, raffinose or stachyose, or a sugar alcohol is a major transporting sugar (Turgeon 1991).

2.2.2.1. Phloem loading of sucrose

Two pathways have been proposed for sucrose transport from mesophyll cells to the sieve element-companion cell complex (SE-CCC) in the minor veins of mature leaves: apoplastic and symplastic phloem loading.

According to the apoplastic loading hypothesis, sucrose passes through plasmodesmata from cell to cell until it reaches the vicinity of the phloem and enters the cell wall (apoplast). Sucrose is taken up into the SE-CCC from the cell wall by a H^+-sucrose symporter (Giaquinta 1983, Evert and Mierzawa 1986). Supporting evidence for the apoplastic loading hypothesis includes the presence of few plasmodesmatal connections between the mesophyll cells and the SE-CCC (van Bel 1992) and the presence of a H^+-sucrose symporter located exclusively on the plasma membrane (Giaquinta 1983, Warmbrodt *et al.* 1989). In addition, recent studies of transgenic plants support the apoplastic loading hypothesis. Transgenic plants expressing the *suc2* gene, encoding a yeast-derived invertase targeted to the apoplast of tobacco (Sonnewald *et al.* 1991), *Arabidopsis* (Sonnewald *et al.* 1991), tomato (Dickinson *et al.* 1991, Lerchl *et al.* 1995), and potato (Heineke *et al.* 1992), showed decreased phloem loading of sucrose, thus supporting the apoplastic loading mechanism. In apoplastic phloem loading plants, sucrose moved symplastically to regions of SE-CCC and then it loaded into the phloem (Beebe and Evert 1992). However, the sucrose effluxing site to the apoplast is not known.

Anatomical studies of *Commelina benghalensis* (van Bel 1986) and *Amaranthus retrofluxus* (Fisher and Evert 1982) showed that there were only a few plasmodesmata between mesophyll cells and the SE-CCC, indicating that an apoplastic pathway is operative. However, the plasmodesmatal frequency did not decrease at all in the sieve element area of *Populus deltoides* (Russin and Evert 1985) and *Triticum aestivum* (Kuo *et al.* 1974). These results indicate that the apoplastic loading is not an universal mechanism due possibly to structural diversities among plant species.

Accumulation of sucrose by the phloem has implicated a role for a H$^+$-sucrose symporter on an energetic basis for active sucrose transport into the SE-CCC. The H$^+$ gradient between the apoplast and the SE-CCC strongly supports the concept that a proton motive force (PMF) provides the energy for sucrose accumulation into the SE-CCC. The high PMF is generated by differences in chemical and membrane potentials between the inside and outside of plasma membrane vesicles (Bush 1989, Bush 1990, Williams *et al.* 1990).

Studies on identification of the H$^+$-sucrose symporter showed two conflicting results. In plasma membranes, a 62 kD protein (Hitz *et al.* 1986, Ripp *et al.* 1988) or a 42 kD protein (Gallet *et al.* 1989), both of which were presumed to be the H$^+$-sucrose symporter, have been identified using immunocytochemical techniques. The two-dimensional pattern of proteins from plasma membrane vesicles showed new polypeptides occurring in a size range between 30 and 55 kDa during a sink-source transition of sugar beet leaves (Frommer *et al.* 1994) and potato tubers (Borgmann *et al.* 1994). However, the exact nature of the H$^+$-sucrose symporter has not yet been identified.

In contrast to the apoplastic loading hypothesis, the symplastic phloem loading hypothesis implies that sucrose moves from mesophyll cells to the SE-CCC via plasmodesmata (Schmitz *et al.* 1987, Madore and Lucas 1987, Turgeon and Wimmers 1988, Turgeon and Gowan 1990, Madore and Webb 1991, Turgeon and Beebe 1991). The symplastic loading of sucrose requires plasmodesmata function as selective and one-way valves (Delrot 1987). It is conceivable that a specialized control system operates in plasmodesmatal connections. Recently, good evidence for the symplastic phloem loading hypothesis has been obtained with a microautoradiography technique from minor veins of *Cucumis* (Schmitz *et al.* 1987), *Ipomoea* (Madore and Lucas 1987), and *Coleus* (Turgeon and Gowan 1990). However, the concept of symplastic loading is highly controversial because it did not successfully ratify the laws of thermodynamics (Turgeon and Beebe 1991).

In conclusion, phloem loading of sucrose has been reported to be apoplastic in some species, symplastic in other species, and both symplastic and apoplastic in still others (Fritz *et al.*

1983, van Bel 1987). The structural diversity among plant species excludes a universal pathway for sucrose movement from photosynthetic tissues to the phloem (Gamalei 1989).

2.2.2.2. Phloem unloading of sucrose

Following long-distance transport through the phloem, sucrose is imported into sinks. Anatomical and physiological studies have shown that sucrose leaves the SE-CCC through plasmodesmata into vascular parenchyma elements (symplastic unloading) (Giaquinta 1983, Ellis *et al.* 1992), directly across the plasma membrane into the apoplast (apoplast unloading) (Wolswinkel 1987, Syndonia Bret-Hart and Silk 1994), or both (Patrick 1990).

Current evidence showed that sucrose was unloaded symplastically in many sink types including expanding leaves (Turgeon 1987), root apices (Giaquinta 1983, Grusak and Minchin 1988), developing seeds (Thorne 1985), and potato tubers (Oparka and Prior 1988). Symplastic unloading of sucrose indicates that there is a concentration gradient of sucrose between the SE-CCC and sink cells and that sucrose transport is affected by a transport capacity of plasmodesmata (Patrick 1990). In contrast, apoplastic unloading of sucrose was found in developing seeds of legumes (Wolswinkel 1992) and the primary root tip of maize (Syndonia Bret-Harte and Silk 1994). Although the apoplastic unloading mechanism of sucrose explains the efflux of sucrose from the SE-CCC to apoplasts, the exact site(s) of sucrose unloading to apoplasts remain to be identified.

2.2.3. Fate of sucrose in sinks

In the cytoplasm of sinks, sucrose is hydrolyzed, at first, to hexoses by invertase (INV) and/or sucrose synthase (SS) (Sung *et al.* 1989, Yelle *et al.* 1991). The principal biochemical pathways of sucrose unloading and carbohydrate utilization are given in Fig. 3. There are two types of invertase in plant cells: acidic INV in the cell wall and vacuole and basic INV in the cytosol. Invertases catalyze the cleavage of sucrose to glucose and fructose which are phosphorylated.

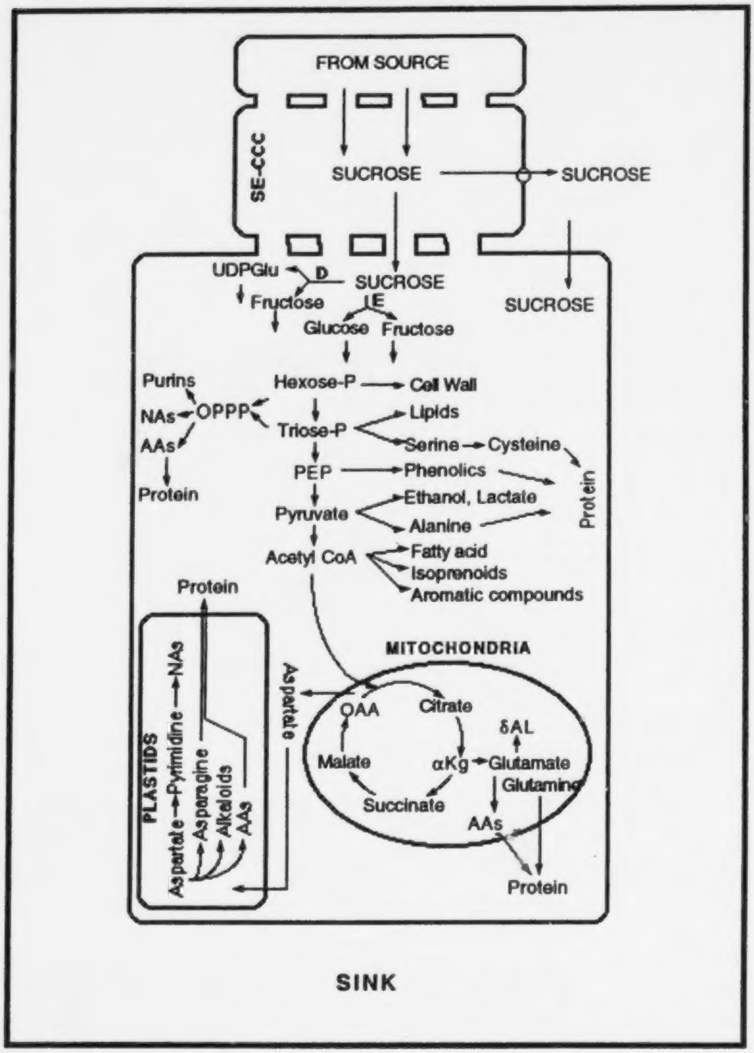
FROM SOURCE
SE-CCC
SUCROSE
SUCROSE
UDPGlu
D
SUCROSE
Fructose
IE
SUCROSE
Glucose
Fructose
Purins
Hexose-P
Cell Wall
NAs
OPPP
Lipids
Triose-P
AAs
Serine
Cysteine
PEP
Phenolics
Protein
Protein
Ethanol, Lactate
Pyruvate
Alanine
Acetyl CoA
Fatty acid
Isoprenoids
Aromatic compounds
Protein
MITOCHONDRIA
PLASTIDS
Aspartate
Pyrimidine
NAs
Asparagine
Alkaloids
AAs
Aspartate
OAA
Citrate
δAL
Malate
αKg
Glutamate
Glutamine
Succinate
AAs
Protein
SINK

Figure 3. Phloem unloading of sucrose and utilization of carbohydrates in the sink. Sucrose moves from the phloem to sinks through plasmodesmata (symplastic unloading) or it moves to sinks after it entered the apoplast by a putative sucrose transporter (o) (apoplastic unloading). In the cytoplasm, sucrose is hydrolyzed by invertase (**E**) and sucrose synthase (**D**). The hexoses and hexose phosphates are metabolized through numerous metabolic processes. Glu, glucose; UDPGlu, UDP glucose; Fru, fructose; Triose-P, triose phosphate; PEP, phosphoenol pyruvate; NAs, nucleic acids; AAs, amino acids; OAA, oxaloacetate; OPPP, oxidative pentose phosphate pathway; αKG, α-ketoglutarate; δAL, δ-aminolevulinate; SE-CCC, sieve-element-companion-cell complex. NOTE: The figure is not intended to show all biosynthetic site locations and, instead, focuses on pathways and source-sink relationships.

Sucrose synthase (SS) is involved in sucrose breakdown to UDP-glucose and fructose. Although the role of INV is still a matter of debate because tissue-specific abundance, compartmentation, and regulation of invertase activities are not well understood (Lerchl 1995), it is not known which pathway is prevalent in plants.

Hydrolysates of sucrose, hexoses, are allocated to various biosynthetic pathways through glycolysis, gluconeogenesis, and the pentose phosphate pathway (Salisbury and Ross 1992, Blakeley and Dennis 1992). Therefore, in the sinks, hexoses are metabolized into a number of carbohydrate pools (hexose phosphate pool, pentose phosphate pool, and glycolytic pathway) before they are used for energy sources through respiration or for building blocks of several structural and functional components of cells through various biosynthesis processes. The first pool of metabolites contains hexose phosphates such as G6P, F6P, and G1P. These metabolites are used for the synthesis of starch and cell wall polysaccharides and for operation of glycolysis and the oxidative pentose phosphate pathway. The second pool is comprised of intermediates of pentose phosphate that are in equilibrium with triose-P. These metabolites are utilized for the biosynthesis of glycerol, nucleic acids, and aromatic amino acids which are precursors of phenolics. The third pool of metabolites is involved in reactions of the glycolytic pathway from glyceraldehyde 3-phosphate to PEP. Of the metabolites in this pool, 3-phosphoglycerate is a precursor of serine, PEP is utilized for aromatic amino acid biosynthesis, pyruvate is a precursor of acetyl CoA for biosynthesis of fatty acids, terpenoids, and several amino acids including alanine. Pyruvate is also used for the TCA cycle that generates energy and precursors for amino acids, porphyrins, alkaloids, and pyrimidines.

2.2.4. Source-sink interactions

Source ability for carbohydrate production and sink demand for carbohydrate utilization in plants are interrelated. There are several comprehensive reviews covering source-sink relationships (Gifford and Evans 1981, Gifford *et al.* 1984, Sonnewald *et al.* 1994). However, the nature of interactions between the source and sinks and the mechanism that controls the interactions are not known.

Sources are powerful organs that support sink growth by a continuous supply of carbohydrates. It is known that a source's ability for carbohydrate production is subject to change by sink demands. Photosynthesis responded positively to increased sink demand for photosynthates (Gifford and Evans 1981). However, not all studies showed that a reduction in sink demand affected the source's ability for carbohydrate production. In cucumber, for example, the rate of photosynthesis of source leaves did not respond to a sink demand (Marcelis 1991). A similar result was reported for *Vicia faba* (Ntsika and Delrot 1986) and *Pisum sativum* (van Oene *et al.* 1992b). These authors found that a reduction in sink strength decreased ^{14}C-assimilate loading into the veins of source leaves and ^{14}C-assimilate export to sink regions. In the source leaves of *Pisum sativum* plants whose sink strength had been reduced, this reduction resulted in an increase in sucrose content, but it did not increase starch content or inhibit photosynthesis (van Oene *et al.* 1992a). These results imply that a reduction of sink strength does not control source ability for carbon fixation or carbohydrate production. Although the effects of sink demand on the source's ability for carbohydrate production are not clearly understood, results from source-sink interaction studies clearly show that sources and sinks interact in regulating carbohydrate translocation.

The source-sink interrelations for sucrose translocation are affected by many metabolic inhibitors. In sources or sinks, inhibition of a specific metabolic reaction by an inhibitor leads to a depletion of particular final products that are core components of plants, or to an accumulation of intermediates that are toxic to plants (Böger 1989, Duke 1990, Moreland 1980). The targets of the inhibitors are in such synthesis processes for amino acids, lipids, carotenoids, chlorophyll, isoprenoids, cellulose, protein, nucleic acids, auxin, and folic acid. Some others inhibit physiological functions of plants such as cell division, respiration, and auxin transport. For example, the herbicide chlorsulfuron inhibits acetolactate synthase in branched-chain amino acid biosynthesis, while the herbicide glyphosate inhibits 5-enol-pyruvyl shikimic acid 3-phosphoate

(EPSP) synthase in aromatic amino acid biosynthesis. These metabolic inhibitors, herbicides, deplete particular final product or cause toxic intermediates to accumulate that can disturb overall metabolism in sources and/or sinks. These metabolic changes result in a reduction of source or sink abilities for carbohydrate production and allocation or for carbohydrate utilization, resulting in a disturbance in source-sink interactions (Geiger and Bestman 1990).

3. MATERIALS AND METHODS

3.1. General

3.1.1. Plant material

Canola (*Brassica napus* L. cv. Westar) seeds[12] were placed in a petri dish containing two sheets of Whatman No. 1 filter paper moistened with distilled water and kept at 21 °C until seeds germinated. Seedlings were transferred to horticultural grade Metro Mix®[13] in 8 x 8 x 8 cm trays. Some seedlings were grown in PVC-covered hard-board boxes containing a modified Hoagland's (1950) nutrient solution with Fe-EDTA (Appendix 1). Seedlings were grown in a growth chamber with conditions of 21/18 °C day/night temperatures, 16-h photoperiod, 400 μE m^{-2} s^{-1} light intensity[14]. The relative humidity was 50%.

3.1.2. Chlorsulfuron

Two formulations of chlorsulfuron, technical product (95% purity) and Glean® (a commercial product, 75% DF), were used in this study. In experiments on absorption and translocation of ^{14}C-sucrose, technical product was used. In other cases, Glean® was used.

Technical product of chlorsulfuron was dissolved in 10 mM Na_2HPO_4-citric acid buffer (pH 8.0) containing 10% (v/v) tetrahydrofuran and 0.1 % (v/v) Cittowet Plus. Glean® was dissolved in distilled water containing 0.1 % (v/v) Agral 90. The technical product or Glean® was applied at various rates depending on the purpose of the experiment.

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[12] A single source of canola seeds, used throughout this study, was supplied by the University of Alberta, Michener Farm. The seeds were harvested in 1989.

[13] Terra-Lite 2000, W. R., Grace & Co. of Canada Ltd.

[14] It was measured with a quantum meter Model LI-185, Li-Cor Inc.

Chlorsulfuron solutions were prepared freshly on the application day because of precipitate formation in aged solutions. Ten µl of chlorsulfuron solution was applied to a certain leaf of seedlings using a micropipet[15]. Six droplets of chlorsulfuron solution were placed on the leaf, avoiding the midvein, or the droplets were spread over the entire leaf surface using the column of the micropipet. The latter application method in which the entire leaf surface was covered with chlorsulfuron solution was used only for ^{14}C-sucrose translocation experiments in sections 3.4 and 3.7 and for sink leaf ability for assimilate import in section 3.6. In other experiments, chlorsulfuron solution was applied as separate droplets.

3.1.3. ^{14}C-sucrose

Appropriate amounts of ^{14}C-sucrose were pipetted into microvials from stock solution [^{14}C(U); sp. act. 23.4 GBq mmol^{-1}; radiochemical purity 99%][16]. After evaporation of solvent, ^{14}C-sucrose was dissolved in potassium phosphate buffer (pH 7.0) with 0.1 % Agral 90 (v/v). ^{14}C-sucrose was applied in the same manner as chlorsulfuron. The applied ^{14}C radioactivity was 834 Bq per seedling.

3.2. Response of canola seedlings to chlorsulfuron, branched-chain amino acids and pantothenic acid, and 2-ketobutyrate

3.2.1. Chlorsulfuron

In a dose-response experiment, six droplets of chlorsulfuron solution with a total volume of 10 µl containing 0.001, 0.01, 0.1, 1, or 10 µg chlorsulfuron per seedling were applied to the first leaves of seedlings at the four-leaf stage. Five days later, leaf area and dry weight of the seedlings were measured.

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[15] Wiretrol 10 µl, Drummond Scientific Co.

[16] NEC-100X, Du Pont Canada Inc. or 11137 ICN Biomedical Canada Ltd.

In time-response experiments, six droplets of chlorsulfuron solution with a total volume of 10 µl containing 1 µg chlorsulfuron per plant were applied to the first leaves of seedlings at the four-leaf stage. Leaf area and dry weight of seedlings were measured and injury symptoms were observed 0, 2, 5, and 8 days after chlorsulfuron treatment.

3.2.2. Supplementation with branched-chain amino acids and pantothenic acid

Canola seedlings were grown in a modified Hoagland's (1950) nutrient solution containing Fe-EDTA. Seedlings at the four-leaf stage were transferred to nutrient solution containing branched-chain amino acids (BCAA), pantothenic acid (PA), or BCAA + PA. The final concentration of BCAA and PA was 5 and 2 mM, respectively. Two days after transfer, the first leaves of seedlings were treated with 1 µg of chlorsulfuron as described in section 3.1.2. Leaf area and dry weight of seedlings were measured 0, 2, 5, and 8 days after treatment. During that period, injury symptoms were recorded. The nutrient solution with BCAA and PA was changed every 2 days.

3.2.3. 2-ketobutyrate

Canola seedlings were grown in nutrient solution as described in section 3.2.2 and the seedlings at the four-leaf stage were transferred to nutrient solution containing 0, 5, 10, 25, or 50 mM 2-ketobutyrate for 3 days. In a further experiment, seedlings were grown in nutrient solution with 50 mM 2-ketobutyrate for 3 days. After the determination of the fresh weight of the seedlings, the total sugar and amino acid contents of the first leaves were determined. The extraction and analysis of total sugar and amino acids are described in section 3.10.

3.3. Source leaf ability to export assimilate

3.3.1. Sink-to-source transition of the first leaves of canola seedlings

Leaf area and dry weight of the first leaves of canola seedlings were measured between 8 and 17 days after transplanting to determine the appropriate source stage for application of chlorsulfuron and ^{14}C-sucrose. During that period, the leaves increased rapidly in size. Sink-to-source transition of the first leaves was determined in accordance with a suggestion by Dale (1992) that such transition occurs when a leaf reaches about a third of its final leaf area or lamina length.

3.3.2. Export of exogenous ^{14}C-sucrose out of the first leaves of intact canola seedlings

During the period of the sink-to-source transition experiment, the ability of the first leaves to export assimilate was determined. Six droplets of solution with a total volume of 10 µl containing 834 Bq ^{14}C-sucrose per seedling were applied to the first leaves of seedlings, and spread over the leaf surface. Twenty-four hours later, ^{14}C activity in plant parts other than the treated leaves was determined as transported ^{14}C. Translocation of exogenous ^{14}C-sucrose and determination of ^{14}C are described in detail in sections 3.4.2 and 3.4.4.

3.4. Translocation of exogenous ^{14}C-sucrose in intact seedlings

3.4.1. Autoradiography

Autoradiographic assay of ^{14}C-sucrose translocation was conducted as described by Crafts and Yamaguchi (1964). Six droplets of chlorsulfuron solution (10 µl total volume) containing 1 µg chlorsulfuron per seedling were applied to the first leaves of seedlings at the four-leaf stage.

Twenty-four hours after chlorsulfuron treatment, six droplets of application solution (10 µl total volume) containing 37 kBq ^{14}C-sucrose per plant were applied to the same leaves to which chlorsulfuron was applied. Twenty-four hours after treatment, root parts were carefully washed with water. Seedlings were freeze-dried, re-humidified, flattened, and then autoradiographed using X-ray film[17]. Three days after exposure, the films were developed in Kodak developer[18] for 3 minutes and fixed in Kodak fixer[19] for 5 minutes.

3.4.2. Application of chlorsulfuron and ^{14}C-sucrose to the same leaf

Absorption and translocation of ^{14}C-sucrose were determined as described by Devine *et al.* (1990). The first leaves of seedlings at the four-leaf stage were treated with 1 µg of chlorsulfuron. Six droplets of chlorsulfuron solution with a total volume of 10 µl per plant were placed on the treated leaf, and spread over the surface. One, 6, 12, or 24 hours after chlorsulfuron treatment, chlorsulfuron-treated leaves were treated with 834 Bq of ^{14}C-sucrose in the same manner as the chlorsulfuron treatment. Twenty-four hours after ^{14}C-sucrose treatment, seedlings were divided into five parts: ^{14}C-sucrose-treated leaf, adjacent leaf (second leaf), shoot apex containing the third and fourth leaves, roots, and remainder including two cotyledonary leaves and stem. The ^{14}C-sucrose-treated leaves were excised and washed three times with 5 ml of 10% ethanol (v/v) per time in liquid scintillation vials[20] to determine unabsorbed ^{14}C activity. The leaves were washed for 30 sec each time by gentle shaking of the vials. Ten ml of EcoLite™ scintillation liquid was added to the leaf washes. Absorption of ^{14}C-sucrose was determined after measuring the ^{14}C activity of the leaf washes using liquid scintillation spectrometry (LSS), and was expressed as percentage of ^{14}C-sucrose applied [(^{14}C found in plants/total ^{14}C applied) x 100].

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[17] X-OMAT AR, Eastman Kodak Co.

[18] Kodak rapid developer, Eastman Kodak Co.

[19] Kodak rapid fixer, Eastman Kodak Co.

[20] Fisherbrand® 20 ml Polyethylene Vials, Fisher Scientific Co.

After determination of absorption, the harvested plant parts were dried at 65 °C for 2 days prior to combustion in a biological sample oxidizer. The oxidation of plant materials and determination of ^{14}C activity are described in section 3.4.4. Translocation was expressed as percentage of ^{14}C recovered [(^{14}C found in other plant parts than the treated leaf/total ^{14}C recovered) x 100].

3.4.3. Application of chlorsulfuron and ^{14}C-sucrose to the adjacent leaves

Experiments were performed in the same manner as described in section 3.4.2. However, chlorsulfuron and ^{14}C-sucrose were applied to separate adjacent leaves. Chlorsulfuron was applied to the first leaves of canola seedlings at the four leaf-stage. One, 6, 12, or 24 hours after chlorsulfuron treatment, ^{14}C-sucrose was applied to the adjacent, separate leaves of the same seedlings. Absorption and translocation of ^{14}C-sucrose were determined as described in section 3.4.2.

3.4.4. Sample combustion and liquid scintillation spectrometry

Plant samples containing ^{14}C were combusted in a biological sample oxidizer[21] using ^{14}C-cocktail[22] for trapping the gaseous $^{14}CO_2$ formed during the combustion process. Sample combustion time was 3 min and, depending on sample size, samples were divided and oxidized separately. Radioactivity was measured by liquid scintillation spectrometry[23]. Efficiency of combustion and ^{14}C recovery was determined by combusting and counting a known amount of ^{14}C-sucrose on filter paper. Efficiency exceeded 95%.

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[21] Biological Oxidizer OX300, R. J. Harvey Instrument Co.

[22] Carbon-14 Cocktail, R. J., Harvey Instrument Co.

[23] MINAXIB Tri-Carb® 4000 Series, Canberra Packard Canada

3.5. Exudation of sucrose out of excised leaves

Six droplets of chlorsulfuron solution with a total volume of 10 μl containing 1 μg chlorsulfuron per seedling were applied to the first leaves of seedlings at the four-leaf stage. The treated leaves were excised using scalpels. The cut petiole tips were put in small petri dishes (i.d. 5.5 cm) containing exudation buffer [5 mM potassium phosphate buffer (pH 6.0) with 1 mM ethylenediaminetetra-acetic acid (EDTA)]. The petiole tips were re-cut after 30 min and placed in microcentrifuge tubes[24] containing 1.5 ml of exudation buffer. The excised leaves were put in a perspex chamber which was kept at 100% relative humidity by a continuous supply of humidified air. The leaves were transferred to fresh exudation buffer every 2 hours for 8 hours. The exudates were freeze-dried and redissolved in 1 ml distilled H_2O. The contents of glucose, fructose, and sucrose of exudates were measured using a combined enzymatic-UV method. The analytical procedures are described in section 3.11.2.

Other exudation experiments were conducted in a controlled environment. The conditions for preparation of excised leaves were described previously in this section. The excised leaves were put in centrifuge tubes (12 x 75 mm) containing 1.5 ml exudation buffer [5 mM phosphate buffer solution with 1 mM EDTA]. Each pair of excised leaves from control and chlorsulfuron-treated seedlings was placed in two identical glass cuvettes (Bassi and Spencer 1979) to exude sugars for 8 hours. The excised leaves were exposed to 350 $\mu l\ l^{-1}$ or 500 $\mu l\ l^{-1}$ CO_2 under 400 $\mu E\ m^{-2}\ s^{-1}$ light at 25 °C. The sucrose or total sugar contents in exudate were determined as described in sections 3.10.2.1 and 3.11.2. Net carbon exchange and transpiration rates were also measured during the same period as described in section 3.8.2.

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[24] Fisherbrand Flat Top Microcentrifuge Tubes 1.5 ml Polypropylenes, Fisher Scientific Co.

3.6. Sink leaf ability to import assimilate

3.6.1. Sink-to-source transition of the third leaves of canola seedlings

Leaf area and dry weight of the third leaves of canola seedlings were measured between 9 and 17 days after transplanting to determine the appropriate sink stage for application of chlorsulfuron and ^{14}C-sucrose. During that period, the leaves increased in size rapidly. Sink-to-source transition of the third leaves was determined as Dale (1992) has suggested (see section 3.3.1 on page 31)

3.6.2. Plant modification

Seedlings at the four-leaf stage were surgically modified to have one source and one sink. The second, the fourth, and two cotyledonary leaves were excised using scalpels[25] and the stem was girdled at the soil line using steam. Therefore, seedlings had only fully expanded first leaves and unexpanded third leaves.

3.6.3. Uptake of exogenous ^{14}C-sucrose by leaf discs

3.6.3.1. Production of leaf discs

After a 2-day recovery period from possible shock, the third leaves of modified seedlings were treated with six droplets of chlorsulfuron solution (10 µl total volume) containing 0.25 µg of chlorsulfuron per seedling. The leaf surface was covered with chlorsulfuron solution. Twenty-four hours after chlorsulfuron treatment, the abaxial side of leaves was abraded with Carborundum[26], washed with distilled water, and the leaf was excised using a scalpel. Leaf discs were made using a sharp cork borer (i.d. 7 mm) and were washed three times in base medium [10 mM 2-[N-morpholino]ethane-sulfonic acid (MES), pH 5.5, 5 mM $CaCl_2$, 2 mM KCl, 1 mM $CaSO_4$ $2H_2O$, 10 mM dithiothreitol (DTT)].

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[25] Surgical Blade No 20, Feather Safety Razor Co. Ltd

[26] 320 Grit-Powder, Fisher Scientific Co.

3.6.3.2. ^{14}C-sucrose uptake

^{14}C-sucrose uptake was executed according to Pitcher *et al.* (1991). The leaf discs were placed in 50-ml beakers containing 20 ml of uptake solution (base medium plus 25 mM sucrose, 150 mM sorbitol, and 150 mM mannitol) with 100 kBq ^{14}C-sucrose. The beakers were shaken at 55 rpm on a gyrotary shaker[27] at 23 °C under 400 $\mu E\ m^{-2}\ s^{-1}$ light. After a 30-min uptake period, the uptake solution was removed by aspiration. Leaf discs were rinsed three times with cold base medium (4 °C) to remove ^{14}C-sucrose in the apoplast and oxidized in a biological oxidizer. Radioactivity was determined as described in section 3.4.4.

3.6.4. Numbers of cells extracted from the third leaves of canola seedlings

Cell numbers were determined by counting protoplasts of the leaves. Protoplasts were isolated according to Robertson and Earle (1986). After a 2-day recovery period from possible shock, the third leaves of modified seedlings were treated with six droplets of chlorsulfuron solution (10 µl total volume) containing 0.25 µg of chlorsulfuron per seedling. The leaf surface was covered with chlorsulfuron solution. Twenty-four hours after chlorsulfuron treatment, the leaves were scored with parallel 1 mm cuts using a scalpel under SCM (0.5 M sorbitol, 10 mM $CaCl_2$, 5 mM MES, pH 5.8) solution. The abaxial side of the leaves was submerged under the enzyme solution (2% Cellulysin[28], 1% Macerozyme[29] in 1 mM MES, pH 5.6, 0.08 M $CaCl_2$, 0.2 M mannitol,1 mM KH_2PO_4). The leaves were incubated in petri dishes (35 mm), and shaken on a gyrotary shaker (55 rpm) for 6 h at 25 °C. When digestion was completed, petri dishes were swirled to release protoplasts. The solution containing protoplasts was removed with disposable pipets to a 167-μ mesh funnel over 15-ml conical centrifuge tubes. The tubes were centrifuged at 55 *g* for 5 min. The pellets were resuspended in 3 ml solution (0.5 M sucrose, 5 mM MES, pH 5.8). The SCM solution 0.5 ml was added on top of the solution. The tubes were centrifuged at 55 *g* for 5 min. The floating band is removed, resuspended in 3 ml SCM solution, and centrifuged at 55 *g* for 5 min. The pellet was resuspended in 100 μl SCM solution. The number of protoplasts was determined with a Füchs-Rosenthal hemocytometer. Photographs of the protoplasts were taken under white light using a Zeiss MC 63 fluorescence microscope[30].

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[27] New Brunswick Scientific Co.
[28] Cellulase onozaka R-10, Yakurt Honsha Co. Ltd.
[29] Macerase® Pectinase, Calbiochem.

3.6.5. Leaf size of the third leaves of modified seedlings

The third leaves of modified seedlings were treated with six droplets of chlorsulfuron solution (10 μl total volume) containing 0.25, 0.5, or 1 μg of chlorsulfuron per seedling to examine whether direct application of chlorsulfuron to sink leaves reduces sink size. The leaf surface was covered with chlorsulfuron solution. Twenty-four hours after treatment, the treated leaves were harvested and dried for 2 days at 65 °C. Dry weight of the leaves was determined.

3.7. Import of exogenous ^{14}C-sucrose into the third leaves of modified canola seedlings

After a 2-day recovery period from possible shock, six droplets of chlorsulfuron solution (10 μl total volume) containing 0.25 μg of chlorsulfuron per seedling were applied to the third leaves of modified seedlings. The leaf surface was covered with chlorsulfuron solution. Twenty-four hours after chlorsulfuron treatment, 834 Bq of ^{14}C-sucrose was applied to the first leaves of seedlings in six droplets of solution. In one experiment, seedlings were harvested 24 hours after

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[30] Carl Zeiss Canada Ltd.

^{14}C-sucrose treatment. Absorption and translocation of ^{14}C-sucrose were determined as described in section 3.4.2. In another experiment, the ^{14}C-sucrose-treated leaves were excised, their cut peticle tips were placed in buffer solution in microcentrifuge tubes, and they were allowed to exude ^{14}C-sucrose for 8 hours as described in section 3.5. The buffer solution was transferred to liquid scintillation vials containing 10 ml of EcoLite™ scintillation liquid. The radioactivity was determined using liquid scintillation spectrometry as described in section 3.4.4.

3.8. Photosynthesis

3.8.1. Chlorophyll content

The first leaves of seedlings at the four-leaf stage were treated with six droplets of chlorsulfuron solution (10 µl total volume) containing 1 µg of chlorsulfuron per seedling. One, 2, 3, and 4 days after treatment, leaf discs were made with a cork borer as described in section 3.6.3.1. The leaf discs were put in disposable culture tubes (12 x 75 mm) containing 7 ml of dimethylsulfoxide (DMSO). Chlorophyll was extracted by incubating the tubes in a water bath at 65 °C for 30 min. The extract was transferred to 10-ml graduated cylinders and made up to 10 ml with DMSO. The absorbance of the extract was measured at 645 and 663 nm using a UV-Vis spectrophotometer[31]. The chlorophyll content was calculated using the equations of Arnon (1949).

3.8.2. Net carbon exchange and transpiration rates

The leaf preparation was described previously in section 3.5. In one experiment, maximum net carbon exchange (NCE) rates of leaves were measured to determine their response to high CO_2. Excised leaves were exposed to 350 µl l^{-1}, 500 µl l^{-1}, or 900 µl l^{-1} CO_2 for 8 hours under 400 µE m^{-2} s^{-1} light at 25 °C. The light was provided by two 400-W high pressure sodium lamps. The temperature inside the chamber was controlled by water circulation. High concentrations of CO_2 were produced by mixing CO_2-free air and compressed air containing 10% CO_2[32]. The NCE rate was measured every 12 min using an infra-red gas analyzer (IRGA)[33] interfaced with a computer system[34]. After 8 hours, the leaf area of the excised leaves was measured using a leaf area meter[35]. The NCE and transpiration rates were calculated. The first data were collected at 0900 hours. In other experiments, the excised leaves were exposed to 350 or 500 µl l^{-1} CO_2 and their NCE and transpiration rates were measured. Starch, total sugar, and amino acid contents of the excised leaves were determined as described in section 3.10.

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[31] Spectronic 601, Baush & Lomb

3.9. Respiration

This experiment was performed as described in 3.8.2, but in darkness. The first leaves of canola seedlings at the four-leaf stage were treated with six droplets of chlorsulfuron solution (10 µl total volume) containing 1 µg of chlorsulfuron per seedling. Twenty-four hours after treatment, the treated leaves were excised. The cut petiole tips of the excised leaves were placed in buffer solution and the leaves were put in cuvettes as described in section 3.8.2. NCE rates of the excised leaves at ambient CO_2 condition were determined as net respiratory CO_2 exchange under darkness. The cuvettes were covered with three layers of black vinyl bags to prevent photosynthesis of the leaves.

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[32] Union Carbide Canada Ltd.

[33] LI-6262 CO_2/H_2O analyzer, Li-Cor Inc.

[34] This sytem was developed by Drs. Bestman, H. D. and Vanden Born, W. H. For detailed information on this system, contact Dr. Vanden Born, W. H., Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada T6G 2P5

[35] LI-3100 Area Meter, Li-Cor Inc.

3.10. Extraction, separation, and analysis of various fractions from plant tissues

3.10.1. Extraction

The first leaves of seedlings at the four-leaf stage were treated with six droplets of chlorsulfuron solution (10 µl total volume) containing 1 µg of chlorsulfuron per plant. Twelve, 24, or 48 hours after treatment, the leaves were frozen in liquid nitrogen, lyophilized and stored at -20 °C until extraction. The extraction was done according to Dickson (1979). The leaves were homogenized with 2 ml MCW (methanol:chloroform:water, 12:5:3, v/v/v) solution with a mortar and pestle. The extractant was put in disposable culture tubes (A) (12 x 75 mm). The pestle and mortar were washed two times with 1.5 ml MCW solution. The solution was combined into tubes A. The tubes were centrifuged at 156 *g* for 10 min. The supernatant was saved in another centrifuge tube. The pellet in tubes A was washed with 5 ml MCW solution. The tubes were centrifuged at 156 *g*. This procedure was repeated until the supernatant was clear. After the final centrifugation, the pellets in tubes A were used for starch analysis (see section 3.10.2.3). Three ml of water was added for every 5 ml of supernatant. The tubes containing the supernatant and water mixture were centrifuged at 156 *g* for 10 min. After centrifugation, the supernatant-water mixture had divided into two layers, a methanol-water phase on top and a chloroform phase on the bottom. The bottom phase containing lipids and pigments was discarded. The top phase was transferred to 100-ml round bottom flasks and evaporated using a rotary evaporator[36] at 35 °C. The final volume of the concentrate was diluted with water to 1 ml. This was stored at -20 °C and used for analysis of sugar (section 3.10.2.1) and amino acids (section 3.10.2.2).

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[36] Rotavapor R110, Büchi

3.10.2. Analysis

3.10.2.1. Total sugar

Total sugar was assayed by the method of Spiro (1966). The anthrone reagent solution was prepared by dissolving 0.4 g anthrone in 16 ml of 95% ethanol, 60 ml of water, and 200 ml of sulfuric acid. A 50-µl aliquot of sample (section 3.10.1) was combined with 2 ml of reagent solution in 6-ml culture tubes. The tubes were capped with lids containing several pinholes, incubated in a water bath at 95 °C for 10 min, and cooled for 30 min at room temperature. The absorbance was measured at 620 nm using a UV-Vis spectrophotometer. A standard curve was prepared with 5.56 mM glucose[37] in each run to convert absorbance units to concentration.

3.10.2.2. Free amino acids

Free amino acids were assayed by the method of Moore (1968). A 40-µl aliquot of sample (section 3.10.1) was combined with 360 µl ninhydrin reagent solution[38] in 6-ml disposable culture tubes. The tubes were capped with lids containing several pinholes, incubated in a water bath at 95 °C for 10 min, and cooled for 30 min at room temperature. A mixture of water: 1-propanol (1:1, v/v) 1.6 ml was added to the tubes. The absorbance was measured at 570 nm using a UV-Vis spectro-photometer. A standard curve was prepared with 2 mM leucine in each run to convert absorbance units to concentration.

3.10.2.3. Starch

A Pharmacia PD-10 (Sephadex® G-25M) column was washed with 25 ml of sodium acetate buffer (pH 4.5). An aliquot of amyloglucosidase [E.C. 3.2.1.3] solution[39] was pipetted on to the column to remove the ammonium ion in the enzyme solution. The enzyme was eluted through the column with 3.5 ml of sodium acetate buffer.

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[37] 635-100, Sigma Chemical Co.

[38] N 1632, Sigma Chemical Co.

[39] A 3042, Sigma Chemical Co.

Two ml of water was added to tubes containing a starch pellet (section 3.10.1). The tubes were capped with lids containing several pinholes, boiled in a water bath for 60 min, and cooled at room temperature for 30 min. A 10-µl volume of amyloglucosidase enzyme solution (61 U) was added. The tubes were incubated at 37 °C for 24 h.

Starch-derived glucose content was determined by an enzymatic method[40]. A 10-µl sample was added to 2 ml of PGO (peroxidase, glucose oxidase, *o*-dianisidine) mixture in 6-ml disposable culture tubes. The tubes were incubated at 37 °C for 30 min. The absorbance was measured at 470 nm. A standard curve was prepared with 5.56 mM glucose in each run to convert absorbance units to concentration.

3.11. Extraction, separation, and analysis of sucrose and hexoses

3.11.1. Extraction

The first leaves of canola seedlings at the four-leaf stage were treated with six droplets of chlorsulfuron solution (10 µl total volume) containing 1 µg of chlorsulfuron per plant. Twelve, 24, or 48 h after treatment, the leaves were extracted as described in section 3.10.1.

3.11.2. Analysis

Sugar content was assayed by the method of Jones *et al.* (1977). Hexokinase[41] [EC 2.7.1.1; HK], glucose-6-phosphate dehydrogenase[42] [EC 1.1.1.49; G6PD], and phosphogluco isomerase[43] [EC 5.3.1.9; PGI] were centrifuged at 1000 *g* for 10 min to remove ammonium sulfate. The precipitates were dissolved in HEPES buffer (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), pH 7.0. Invertase[44] [EC 3.2.1.26; INV] was dissolved directly in HEPES buffer (pH 7.0).

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[40] 510-DA, Sigma Chemical Co.

[41] H 5625, Sigma Chemical Co.

[42] G 5760, Sigma Chemical Co.

A 100-µl aliquot of sample (section 3.11.1) was added to 1 ml of reaction buffer [0.1 M HEPES, 0.4 mM ß-nicotinamide adenine dinucleotide phosphate (NADP), 1 mM adenosine 5'-triphosphate (ATP), 5 mM $MgCl_2$, and 0.5 mM dithiothreitol (DTT)]. After the addition of HK (0.7 U) and G6PD (7.5 U) to the buffer solution, the tubes were incubated for 30 min at 23 °C. The absorbance was measured before and after HK and G6PD were added. After addition of PGI (2.5 U) to the buffer solution, the tubes were incubated for 30 min at 23 °C. The absorbance was measured. For sucrose determination, INV (20 U) was added to 100 µl sample and incubated at 23 °C for 30 min. The absorbance at 340 nm was measured before and after INV was added. After HK, G6PD, and PGI were added as described for hexoses, the absorbance was measured at 340 nm. A standard curve was prepared with 5.56 mM glucose in each run to convert absorbance units to concentration.

3.12. Protein extraction, quantification, and separation

3.12.1. Extraction

The first leaves of canola seedlings at the four-leaf stage were treated with six droplets of chlorsulfuron solution (10 µl total volume) containing 1 µg of chlorsulfuron per plant. Proteins were extracted from the leaves 0, 12, 24, or 48 h after chlorsulfuron treatment. The leaf extraction procedure was modified from Mohapatra *et al.* (1987). The leaves were frozen in liquid nitrogen, lyophilized, and homogenized with 5 ml homogenizing buffer (Tris[45]-HCl, pH 7.5, 10 mM KCl, 20 mM $MgCl_2$, and 5 mM mercaptoethanol) using a mortar and pestle on ice. The homogenate was filtered though two layers of moistened Miracloth®[46] and collected in a centrifuge tube. The mortar and pestle were washed with 5 ml homogenizing buffer. The buffer solution was filtered though the same Miracloth®. The tubes were centrifuged[47] at 20,000 *g* , 4 °C, for 30 min. Supernatant (A) was decanted in another centrifuge tube and kept on ice. The pellet was resuspended with 5 ml homogenizing buffer. After centrifugation of the tubes again at 20,000 *g*, 4 °C, for 30 min, the supernatant was combined with A. The supernatant was used for protein quantification (section 3.12.2) and profile in SDS-PAGE (section 3.12.3) experiments.

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[43] P 5381, Sigma Chemical Co.

[44] I 9253, Sigma Chemical Co.

[45] [tris-(hydroxymethyl)aminomethane]

3.12.2. Quantification

Protein content was determined according to the method of Bradford (1976). A 10-µl aliquot of sample (section 3.12.1) was combined with 5 ml Bradford reagent (0.01% Coomassie Brilliant Blue G-250, 4.7% ethanol, and 8.5% phosphoric acid) in 6-ml culture tubes. The tubes were kept at room temperature for 10 min. Absorbance was measured at 595 nm using a UV-Vis spectrophotometer. Each time the assay was run, a standard curve was prepared with bovine serum albumin[48] to convert absorbance units to protein concentration.

3.12.3. Polyacrylamide gel electrophoresis

Thirty ml of acetone were added to supernatant A in centrifuge tubes (section 3.12.1). The tubes were stored at -20 °C for 1 h and centrifuged at 20,000 *g* for 10 min at 4 °C. The supernatant was discarded. After desiccation under vacuum, the pellets were dissolved with 50 µl of sample buffer [0.5 M Tris-HCl, pH 6.8, glycerol, 10% (w/v) SDS, 2-ß-mercaptoethanol, 0.05% (w/v) bromophenol blue]. The buffer solution was transferred to 1.5-ml centrifuge tubes and boiled at 95 °C for 4 min. The tubes were centrifuged again at 20,000 *g* for 5 min at 4 °C. The supernatant was used for SDS-PAGE.

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[46] Calbiochem
[47] Sorvall RC-5B Refrigerated Super Centrifuge, Du Pont Instrument
[48] A 7906, Sigma Chemical Co.

SDS-PAGE procedures were modified from Weber and Osborn (1969). Running gel[49] solution was prepared in a 100-ml beaker without N,N'-methylene-bis-acrylamide and N,N,N',N'-tetramethylethylenediamine (TEMED) and degassed under vacuum. N,N'-methylene-bis-acrylamide and TEMED were then added. The solution, which contained a total 7.5% SDS, was poured between glass plates of the Hoefer Scientific Instruments' SE600 vertical slab gel electrophoresis unit and allowed to polymerized. Stacking gel[50] solution was prepared according to the same procedure as the running gel and poured on top of the running gel. A comb was placed in the stacking gel during polymerization to form wells. The wells were filled with running buffer solution[51], pH 8.3. Aliquots of samples and SDS-PAGE molecular weight markers[52] were loaded into wells filled with the running buffer using a microsyringe[53]. The glass plates were returned to the gel box. The upper and lower buffer chambers were filled with running buffer. Electrophoresis was conducted at a constant current of 35 mA per gel for 4 h. After electrophoresis, gels were removed from glass plates and stained overnight in a staining solution (0.1% Coomassie Brilliant Blue R250, 40% methanol, and 10% acetic acid). The stained gel was destained in a mixture of 40% methanol and 10% acetic acid until the background of the gel was cleared.

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[49] Running gel (0.375 M Tris, pH 8.8) was made from 25 ml 1.5 M Tris-HCl (pH 8.8), 1 ml 10% (w/v) SDS stock solution, 50 ml 30% N,N'-methylene-bis-acrylamide stick solution, 0.5 ml 10% ammonium persulfate, 50 µl TEMED in total volume of 100 ml.

[50] Stacking gel (0.125 M Tris, pH 6.8) was composed of 25 ml 0.5 M Tris-HCl (pH 6.8), 1 ml 10% (w/v) SDS stock solution, 13 ml 30% N,N'-methylene-bis-acrylamide stock solution, 0.5 ml 10% ammonium persulfate, 0.1 ml TEMED in total volume of 100 ml.

[51] Running buffer was composed of 15 g Tris base, 72 g glycine, and 5 g SDS per 1 liter.

[52] SDS-6H, High Molecular Weight Standard Mixture, Sigma Chemical Co.
or 161-0317, SDS-PAGE Standards, Bio-Rad.

[53] Microliter #750, Hamilton Co. Inc.

3.13. Extraction and analysis of nitrate reductase, phosphoenolpyruvate carboxylase, and sucrose phosphate synthase

The first leaves of canola seedlings at the four-leaf stage were treated with six droplets of chlorsulfuron solution (10 µl total volume) containing 1 µg of chlorsulfuron per seedling. The treated leaves were used for extractions of nitrate reductase, phosphoenolpyruvate carboxylase, and sucrose phosphate synthase.

3.13.1. Nitrate reductase

Chlorsulfuron-treated leaves were abraded with Carborundum[54] for 15 s, washed with water, and excised using a scalpel. In one experiment, 0, 6, 12, 18, 21, or 24 h after chlorsulfuron treatment, leaf discs were made as described in section 3.6.3.1. During the dark period, the leaf discs were prepared under near darkness to avoid light-induced NR activation. The leaf discs were put in 125-ml Erlenmeyer flasks containing 10 ml of 1 mM potassium phosphate buffer (pH 7.5) and 0.3 M KNO_3 for 1 h. The flasks were put in a water bath and incubated with continuous shaking at 23 °C under darkness. In another experiment, leaf discs were prepared 24 h after chlorsulfuron treatment and incubated as described earlier in this section. However, the leaf discs were incubated for 2 hours.

Nitrate reductase (NR) activity was measured *in vivo* (Sanderson and Cocking 1964). A 1-ml aliquot of supernatant was put in 6-ml disposable culture tubes containing 1% sulfanilamide and 0.02% N-(1-naphthyl)ethylenediamine dihydrochloride. The tubes were incubated at 25 °C for 30 min. The absorbance was measured at 540 nm using a UV-Vis spectrophotometer. The NR activity was determined by conversion of absorbance units to nitrite concentration using a standard curve of 1 mM KNO_2.

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[54] 320 Grit-Powder, Fisher Scientific Co.

3.13.2. Phosphoenolpyruvate carboxylase

3.13.2.1. Extraction

Phosphoenolpyruvate carboxylase (PEPcase) was extracted according to Siegl and Stitt (1990). Leaves were frozen in liquid nitrogen, lyophilized, and homogenized in a mortar prechilled with liquid nitrogen in 1 ml of extraction buffer [50 mM HEPES-KOH, pH 7.4, 12 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 1 mM EDTA, 1 mM DTT, 10% glycerol, 2 mM benzamidine, 2 mM ε-amino-η-caprionic acid]. The supernatant was filtered through two layers of Miracloth® into centrifuge tubes.

3.13.2.2. Assay

PEPcase activity was measured immediately spectrophotometrically by coupling the reaction to NADH-oxidation mediated by malate dehydrogenase [EC 1.1.1.37] according to Stitt *et al.* (1988). Samples were allowed to thaw at 4 °C and the extract was centrifuged at 16,000 *g* for 5 min at 4 °C. The supernatant was desalted with a Sephadex® G-25 column[55] equilibrated with the extraction solution.

A 50-µl aliquot of supernatant was assayed in the presence of reaction buffer (50 mM Tris-HCl, pH 7.6, 0.33 mM $NaHCO_3$, 217 µM NADH, 33 µM $MgCl_2$, 83 µM DTT, 10 units malate dehydrogenase[56], and 217 µM PEP) (1 ml final volume). The reaction was measured at 340 nm with a UV-Vis spectrophotometer connected to a chart recorder. After reaction, oxidation of NADH was expressed in a slope. It was calculated as µmol NADH oxidation per minute.

The V_{max} and K_m of PEPcase in the treated leaves of control seedlings were determined by addition of different concentrations of PEP to the reaction buffer containing a 50-µl aliquot of supernatant.

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[55] PD-10 column, Phamacia Biotech.

[56] M 9004, Sigma Chemical Co.

3.13.3. Sucrose phosphate synthase

3.13.3.1. Extraction

Extraction and assay for sucrose phosphase synthase (SPS) were done according to Walker and Huber (1989). Leaves were frozen in liquid nitrogen, lyophilized, and stored at -20 °C until extraction. The frozen leaves were homogenized in a mortar prechilled with liquid nitrogen in 4 ml of homogenizing buffer (50 mM Mops[57]-NaOH, pH 7.5, 15 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, 0.1% Triton X-100). The homogenate was filtered though two layers of Miracloth® into centrifuge tubes. The tubes were centrifuged at 20,000 *g* for 15 min at 4 °C. The supernatant was desalted immediately on a Sephadex® G-25 column[58] with running buffer (50 mM Mops-NaOH, pH 7.5, 15 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT).

3.13.3.2. Assay

The activity of SPS was assayed both under limiting (low substrate and with Pi) and Vmax (high substrate and without Pi) conditions as F6P-dependent formation of sucrose from uridine 5'-diphosphate glucose (UDPG). Under the limiting conditions, a 45-µl aliquot of sample was combined with 50 mM Mops-NaOH, pH 7.5, 10 mM UDPG, 3 mM F6P, 12 mM G6P (activator), 10 mM Pi (inhibitor), 15 mM $MgCl_2$, and 2.5 mM DTT (total volume of 70 µl) in a 1.5-ml centrifuge tube. The tubes were incubated in a water bath at 25 °C for 15 min. Under the Vmax conditions, the tissue extracts were assayed in the presence of 10 mM F6P, 40 mM G6P, 10 mM UDPG, using identical buffer, volume, and reaction time as described for the limiting conditions. After incubation, 70 µl of 30% KOH was added to terminate the reaction and the tubes were boiled at 95 °C for 10 min. After cooling at room temperature, the supernatants were transferred to 6-ml disposable culture tubes. Two ml of anthrone reagent (section 3.10.2.1) was added and the supernatants were incubated at 40 °C for 20 min. The absorbance was measured at 620 nm using a UV-Vis spectrophotometer. Each time the assay was run, a standard curve was prepared with 1 mM sucrose, to convert absorbance units to concentration.

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[57] 3-[N-Morpholino]propanesulfonic acid

[58] PD-10 Columns, Phamacia Biotech.

4. RESULTS AND DISCUSSION

4.1. Effect of chlorsulfuron on plant growth

Chlorsulfuron-induced phytotoxicity in canola (*Brassica napus* cv. Westar) seedlings was studied in this section. A dose-response experiment (4.1.1) was performed to determine a herbicide dosage for a time-response experiment. The effects of herbicide on plant growth were examined over time in a time-response experiment (4.1.2). Effects of branched-chain amino acids (leucine, valine, and isoleucine) and pantothenic acid, and 2-ketobutyrate were determined to know whether the herbicide-induced phytotoxicity was due to a shortage of branched-chain amino acids and pantothenic acid (4.1.3) or an accumulation of 2-ketobutyrate (4.1.4).

4.1.1. Dose-response of canola seedlings

The first leaves of canola seedlings at the four-leaf stage were treated with different amounts of chlorsulfuron. The leaf area and dry weight of herbicide-treated seedlings were measured 5 days after treatment.

Both leaf area and dry weight of seedlings were reduced by chlorsulfuron treatment at dosages greater than 0.01 µg (Fig. 4). Doses of 0.1 µg, 1 µg, and 10 µg chlorsulfuron reduced leaf area by 33%, 47%, and 54%, respectively. On the basis of these results, canola seedlings were treated with 1 µg of chlorsulfuron to develop phytotoxicity.

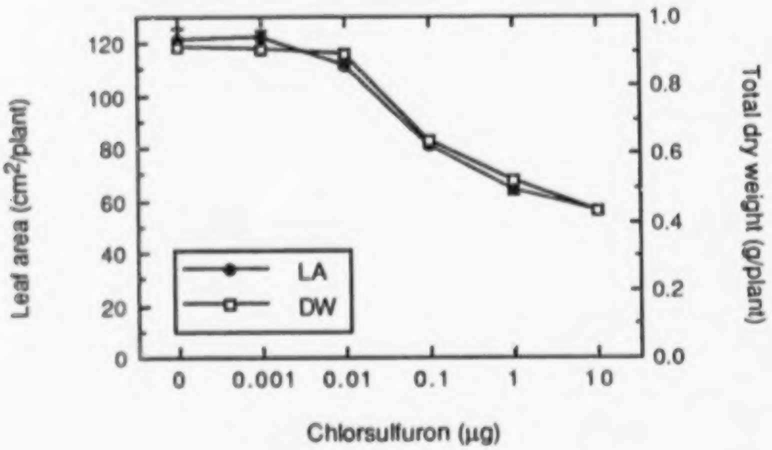

Figure 4. Dose-response of canola seedlings to chlorsulfuron. The leaf area (LA) and the dry weight (DW) of herbicide-treated seedlings were determined 5 days after treatment. Means and standard errors are based on data from two 3-replicate experiments.

4.1.2. Time-response of canola seedlings

The first leaves of canola seedlings were treated with 1 µg of chlorsulfuron. Leaf area and dry weight of control and chlorsulfuron-treated seedlings were measured. In addition, injury symptoms of chlorsulfuron-treated seedlings were observed.

In both control and chlorsulfuron-treated seedlings, total leaf area and dry weight increased with time (Fig. 5). Between 2 and 8 days after treatment, the total leaf area and dry weight of control seedlings increased at the rate of 30 cm^2 and 0.19 g, respectively, per day. That change was due solely to increases in the third and the fourth leaves. The leaf area and dry weight of the first and second leaves, stem, and roots showed little or no change during that period.

The growth rate of chlorsulfuron-treated seedlings was much lower than that of control seedlings (Fig. 5). The increases in the total leaf area and dry weight of the treated seedlings were one third and one half, respectively, of those of control seedlings. Two days after treatment, although the leaf area and dry weight of most plant parts showed no change after that time, the second leaf of chlorsulfuron-treated plants increased in leaf area and dry weight. Eight days after treatment, the leaf area and dry weight of the second leaf of chlorsulfuron-treated plants were 30% and 65% higher, respectively, than they were in control seedlings.

In addition to growth inhibition, chlorsulfuron-treated seedlings showed injury symptoms (Fig. 6). Twenty-four hours after chlorsulfuron treatment, seedlings showed the first injury symptom, chlorosis, in the third and fourth leaves. Downward leaf rolling advanced subsequently in the third leaves. Two days after treatment, anthocyanins appeared around the leaf margins of the second leaves and on chlorsulfuron-treated spots on the first leaves.

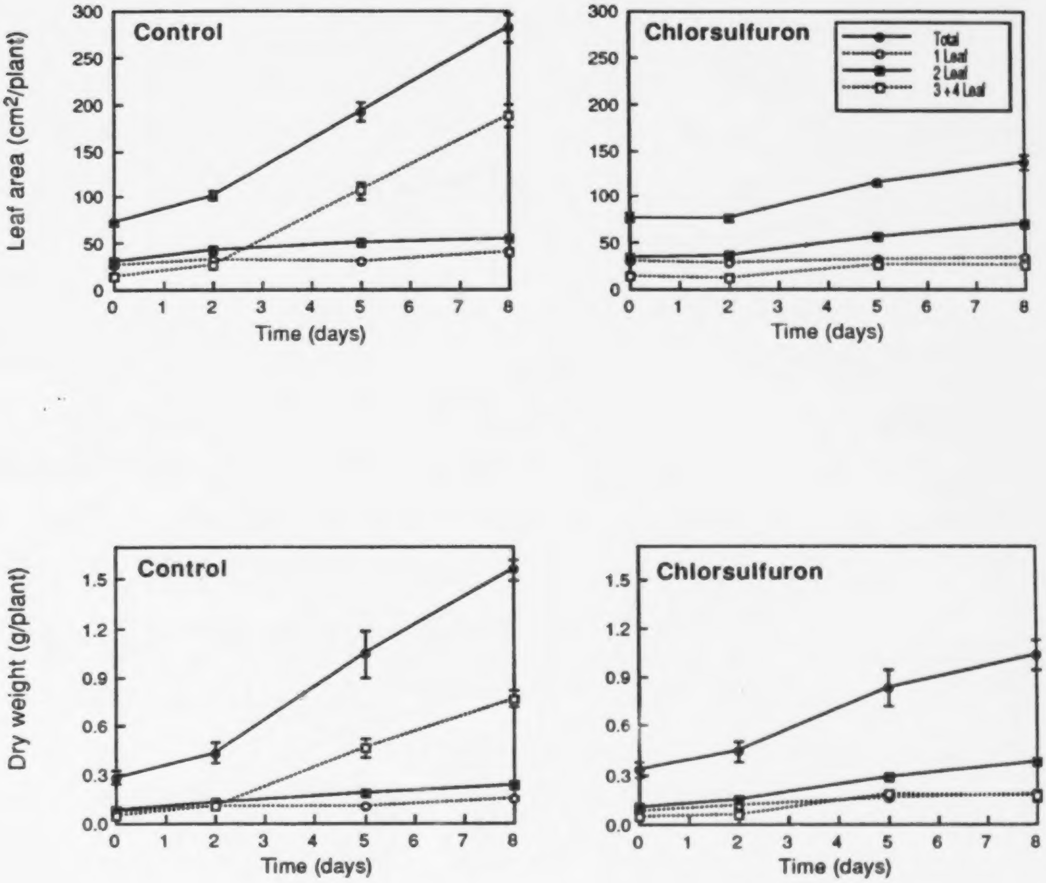

Figure 5. Response of canola seedlings to chlorsulfuron. Total dry weight is comprised of dry weights of the first, second, third, and fourth leaves, stem, and roots. Means and standard errors are based on data from two 2-replicate experiments.

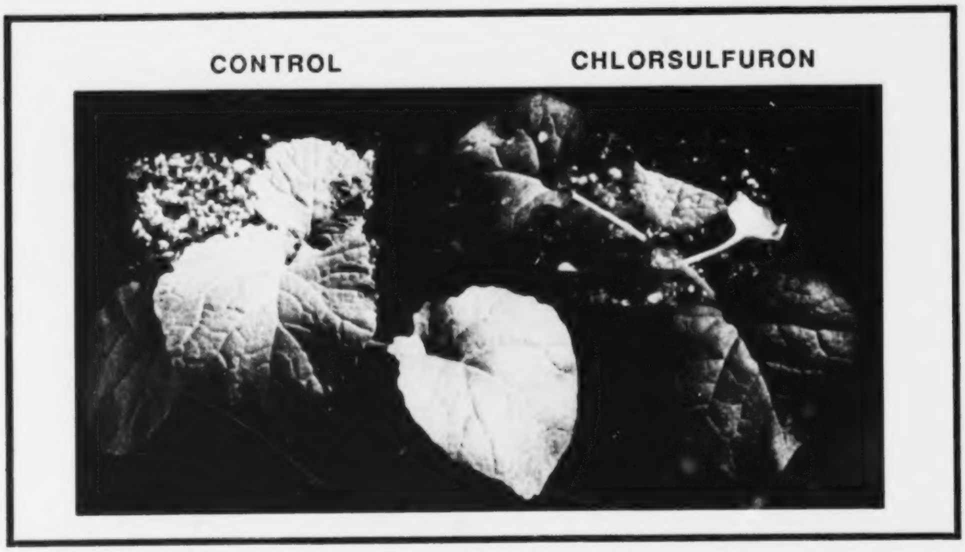
CONTROL
CHLORSULFURON

Figure 6. Injury symptoms in chlorsulfuron-treated canola seedlings. Five days after treatment, the treated seedlings show chlorosis, downward leaf rolling, and anthocyanin accumulation.

4.1.3. Response of chlorsulfuron-treated canola seedlings to supplementation of branched-chain amino acids or branched-chain amino acids plus pantothenic acid

The aim of this experiment was to examine whether supplementation of branched-chain amino acids (BCAA) and pantothenic acid (PA) could prevent chlorsulfuron-induced phytotoxicity in canola seedlings. Before chlorsulfuron treatment, canola seedlings at the four-leaf stage were placed in nutrient solution (Appendix 1) that contained BCAA, PA, or BCAA plus PA for 2 days. Dry weight of the third and fourth leaves (the youngest two leaves) of the seedlings were measured over time.

Two days after supplementation (0 days after chlorsulfuron treatment), dry weight of the third and fourth leaves of control seedlings was 0.038-0.052 g per plant, regardless of supplementation. No injury symptoms were observed in the control seedlings.

Four days after supplementation, dry weight of the third and fourth leaves of control seedlings without supplementation increased at the rate of 0.11 g per day. The control seedlings supplemented with PA had dry weights similar to those of the seedlings without supplementation during 8-day experiments. Supplementation with BCAA or BCAA plus PA, however, partially reduced the growth of the third and fourth leaves: seven days after supplementation, average dry weights of third and fourth leaves supplemented with BCAA or BCAA plus PA were half of those without supplementation (Table 2). In addition, supplementation with BCAA or BCAA plus PA changed the growth pattern in control seedlings. The control seedlings supplemented with BCAA or BCAA plus PA had greener leaves (Fig. 7) and a larger root system (data not shown) than the seedlings without supplementation.

In chlorsulfuron-treated seedlings, growth of the third and fourth leaves was inhibited five days after treatment (Table 2). Supplementation with BCAA partially prevented the chlorsulfuron-induced growth inhibition. Eight days after chlorsulfuron treatment, the dry weight of the third and fourth leaves of the treated seedlings supplemented with BCAA was not different from those in control seedlings supplemented with BCAA, perhaps due to high variability. In

Table 2. Effect of supplementation with branched-chain amino acids and pantothenic acid on dry weights of chlorsulfuron-treated canola seedlings. Canola seedlings were pre-incubated in a nutrient solution containing branched-chain amino acids (BCAA), BCAA plus pantothenic acid (PA), or PA for 2 days. Half of them then were treated with chlorsulfuron (CS). Data are based on two 2-replicate experiments. Values designated by the same letter (columns for each time interval) are not significantly different by LSD$_{0.05}$ tests. *F*-values for the interaction of CS x BCAA at 5 and 8 days after chlorsulfuron treatment were significant at the P$_{0.01}$ level.

	Days After Chlorsulfuron Treatment					
Treatment	2		5		8	
	-CS	+CS	-CS	+CS	-CS	+CS
			(g/plant)			
Control	0.096 ab	0.062 b	0.467 a	0.179 b	0.760 ab	0.160 c
BCAA	0.147 ab	0.086 ab	0.247 b	0.167 b	0.498 b	0.279 bc
BCAA + PA	0.140 ab	0.148 ab	0.242 b	0.236 b	0.446 bc	0.226 bc
PA	0.170 a	0.087 ab	0.434 a	0.135 b	1.046 a	0.203 c

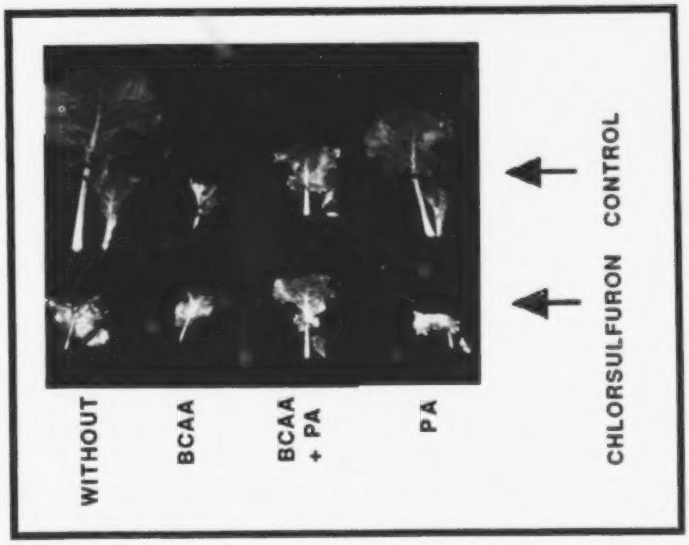
WITHOUT
BCAA
BCAA + PA
PA
CHLORSULFURON
CONTROL

Figure 7. Effect of supplementation with branched-chain amino acids (BCAA), branched-chain amino acids plus pantothenic acid (BCAA + PA), or pantothenic acid (PA) on chlorsulfuron-treated canola seedlings. The third and fourth leaves of chlorsulfuron-treated seedlings with and without supplementation show chlorosis 5 days after treatment. CONTROL, without chlorsulfuron treatment; WITHOUT, without supplementation.

chlorsulfuron-treated seedlings supplemented with BCAA plus PA, *F*-values for the interaction of CS x BCAA x PA at 5 and 8 days after treatment were not significant at the $P_{0.05}$ level, while the *F*-values for the interaction of CS x BCAA at 5 and 8 days after treatment were significant at the $P_{0.05}$ level, indicating that PA did not contribute to preventing the chlorsulfuron-induced growth inhibition in treated canola seedlings.

Chlorsulfuron-treated seedlings showed injury symptoms: chlorosis in the third and fourth leaves, downward leaf rolling in the second leaf, and anthocyanin accumulation in the first and second leaves five days after treatment. In chlorsulfuron-treated seedlings, supplementation with BCAA or BCAA plus PA prevented downward leaf rolling. The downward leaf rolling was not prevented, however, by supplementation with PA, suggesting that it was associated with a shortage of BCAA. Supplementation with BCAA plus PA prevented chlorosis, while supplementation with only BCAA did not prevent it (Fig. 7). The results suggest that PA was involved in the partial prevention of chlorsulfuron-induced phytotoxicity. In chlorsulfuron-treated seedlings, supplementation with BCAA or BCAA and PA, however, did not prevent the accumulation of anthocyanins in the expanded first and second leaves.

In conclusion, chlorsulfuron-induced growth inhibition was partially prevented by supplementation with BCAA; chlorsulfuron-induced injury symptoms also were partially prevented by supplementation with BCAA plus PA.

4.1.4. Effect of 2-ketobutyrate on plant growth

The aim of this experiment was to examine whether exogenous 2-ketobutyrate could induce the phytotoxicity observed in chlorsulfuron-treated canola seedlings.

4.1.4.1. Time-response of canola seedlings

Canola seedlings at the four-leaf stage were placed in nutrient solution (Appendix 1) that contained 0, 5, 10, 25, or 50 mM 2-ketobutyrate, for 3 days. Total leaf area and dry weight of seedlings were reduced 11% and 20%, respectively, by 50 mM 2-ketobutyrate. No such growth inhibition occurred at dosages lower than 50 mM. On the basis of these data, seedlings were treated with 50 mM 2-ketobutyrate in further experiments.

Fresh weights of both control and 2-ketobutyrate-treated seedlings increased over time (Fig. 8). However, the growth rate of treated seedlings was lower than that of control seedlings after 24 h. Seventy-two hours after 2-ketobutyrate treatment, the fresh weight of treated seedlings was 73% of control. No injury symptoms were observed in 2-ketobutyrate-treated seedlings.

4.1.4.2. Effect of 2-ketobutyrate on selected metabolites

In the first leaves of control seedlings, total sugar content at 0 h was 28 nmoles glucose equivalent per mg FW (Fig. 9). It was relatively constant for 48 h and then decreased slightly. In the first leaves of seedlings treated with 2-ketobutyrate, total sugar content was higher than that in control seedlings at the 24-h sampling time but did not differ later.

Free amino acid content in the first leaves of control seedlings at 0 h was 16 nmoles leucine equivalent per mg FW (Fig. 10). Thereafter, the content decreased. In the first leaves of seedlings treated with 2-ketobutyrate, the free amino acid content was the same as in control seedlings for 48 h, but then it decreased to less than half that in controls.

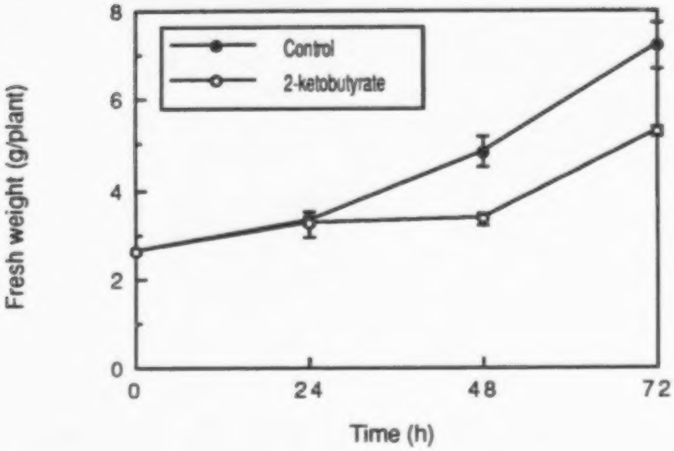

Figure 8. Fresh weight of canola seedlings grown in nutrient solution containing 50 mM 2-ketobutyrate. Means and standard errors are based on data from 4 replicates.

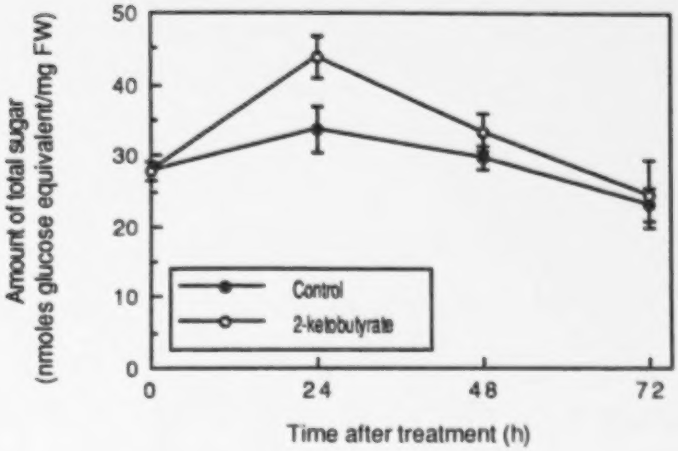

Figure 9. Total sugar content of the first leaves of canola seedlings grown in nutrient solution containing 50 mM 2-ketobutyrate. Means and standard errors are based on data from 4 replicates.

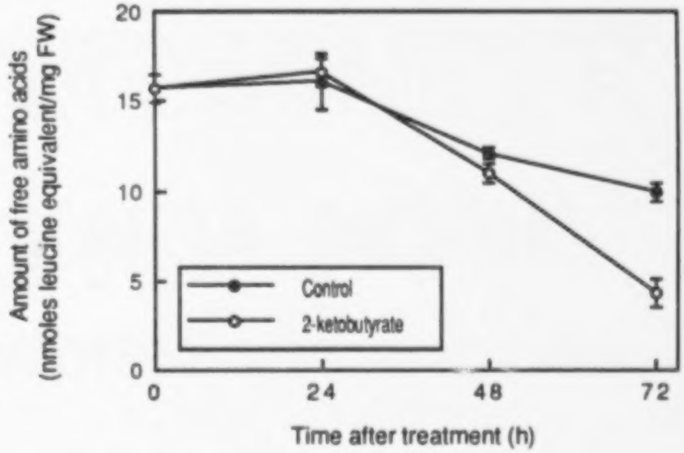

Figure 10. Free amino acid content of the first leaves of canola seedlings grown in nutrient solution containing 50 mM 2-ketobutyrate. Means and standard errors are based on data from 4 replicates.

4.1.5. Discussion

Ray (1980) suggested that shortages of BCAA and PA induced by chlorsulfuron reduced plant growth and eventually lead to death of chlorsulfuron-treated plants. Several researchers (Ray 1984, Scheel and Casida 1985, Mersie and Foy 1987) have reported that supplementation of BCAA fully or partly prevented chlorsulfuron-induced phytotoxicity, supporting Ray's suggestion. Similarly, supplementation with BCAA in this research prevented at least partially the growth inhibition in chlorsulfuron-treated canola seedlings. Although an involvement of PA for chlorsulfuron-induced phytotoxicity had been suggested by Ray (1980), no one has shown that chlorsulfuron depletes the pool size of PA or that a shortage of PA results in the same metabolic and physiological changes as chlorsulfuron treatment. If Ray's hypothesis is correct, then the partial prevention of growth reduction in chlorsulfuron-treated canola seedlings by supplementation with BCAA could be due to a shortage of PA. In valine biosynthesis, 2-oxoisovalerate, a precursor of valine, branches to valine and two intermediates for leucine synthesis, 2-isopropyl malate, and for PA synthesis, 2-dehydropantoate (Anderson and Beadall 1991). Since PA is a core component of coenzyme A that acts as a carrier of acyl groups in many metabolic processes such as fatty acid synthesis and pyruvate oxidation, a shortage of PA caused by chlorsulfuron is expected to change those metabolic processes.

After 7 days' growth, however, chlorsulfuron-treated canola seedlings supplemented with BCAA showed injury symptoms and a partial growth inhibition of the youngest leaves. These results suggest that chlorsulfuron-induced phytotoxicity, growth inhibition and injury symptoms, was not due simply to a shortage of BCAA. In both control and chlorsulfuron-treated canola seedlings, supplementation with BCAA resulted in greener and smaller leaves and larger root systems. The results indicate that supplementation with BCAA decreased the shoot:root ratio in those seedlings. When root growth is predominant, carbohydrates are translocated mainly to roots which results in a shortage of carbohydrate in the actively growing parts of the shoot. Therefore, the growth reduction in the third and fourth leaves of chlorsulfuron-treated canola seedlings supplemented 5 to 8 days with BCAA are suggested to be related to a change in carbohydrate partitioning.

Some researchers have reported that the accumulation of 2-ketobutyrate or 2-aminobutyrate by chlorsulfuron treatment reduces growth of microorganisms and they have suggested that this mechanism might result in growth reduction in plants (LaRossa and Van Dyk 1987, LaRossa *et al.* 1987, Schloss 1989). Reports have indicated that 2-ketobutyrate inhibited cell division in *Allium* (Lanzagorta *et al.* 1988) and *Hordeum* (Reid *et al.* 1985). Although treatment of canola seedlings with 50 mM of 2-ketobutyrate reduced plant growth (Fig. 8), changes in contents of total sugar and free amino acids in the first leaves were different from those in chlorsulfuron-treated plants. Free amino acid and total sugar contents in 2-ketobutyrate-treated seedlings were the same or lower than those in control seedlings, whereas amounts of these metabolites in chlorsulfuron-treated plants were greated than those in control seedlings (Vanden Born *et al.* 1988). A recent study by Shaner and Singh (1993) also showed that growth inhibition by imazapyr, an imidazolinone herbicide that affects the same target enzyme as chlorsulfuron, was not due to an accumulation of 2-ketobutyrate or 2-aminobutyrate in herbicide-treated maize.

In conclusion, chlorsulfuron-treated canola seedlings showed growth inhibition and injury symptoms. In treated seedlings, the growth of the youngest two leaves was inhibited during 8-day experiments. The chlorsulfuron-induced growth inhibition was partially prevented by supplementation with BCAA. The chlorsulfuron-induced injury symptoms were prevented to a greater extent by supplementation with BCAA and PA than by supplementation with BCAA alone. The results from growth inhibition and injury symptoms suggest that chlorsulfuron-induced phytotoxicity is associated with a shortage of BCAA and PA.

4.2. Effect of chlorsulfuron on sucrose transport out of chlorsulfuron-treated leaves of canola seedlings

In this section, the aim was to test whether the reduced growth of chlorsulfuron-treated canola seedlings was associated with reduced translocation of sucrose. Two types of experiments were performed: translocation of exogenous ^{14}C-sucrose and exudation of endogenous sucrose.

4.2.1. Sink-to-source transition of the first leaves of canola seedlings

Leaf area and dry weight of the first leaves of canola seedlings were measured to determine the appropriate growth stage of the source leaf for application of chlorsulfuron and ^{14}C-sucrose. Leaf area increased linearly at the rate of 4.4 cm^2 d^{-1} between 8 and 15 days after transplanting (DATP) (Fig. 11), at which time the maximum leaf area was attained. The pattern of dry weight increase over time was similar to that of leaf area (data not shown). Translocation of ^{14}C out of the first leaves of canola seedlings, following ^{14}C-sucrose application, increased linearly between 8 and 12 DATP (Fig. 11), and then levelled off.

The results from this experiment suggest that a sink-to-source transition of the first leaves of canola seedlings occurred between 9 and 10 DATP, in accordance with Dale's suggestion (1992) that sink-to-source transition occurs when a leaf reaches about a third of its final leaf area or lamina length. The sucrose translocation results imply that the first leaves predominantly import assimilates before 9 DATP, balance the import and export of assimilates between 9 and 10 DATP, and favor the export of assimilates after 11 DATP. Therefore, chlorsulfuron and ^{14}C-sucrose were applied between 11 and 12 DATP.

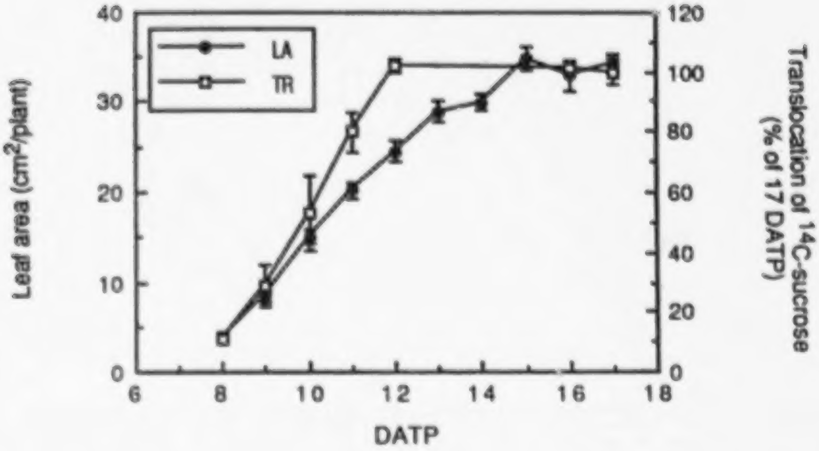

Figure 11. The sink-to-source transition of the first leaves of canola seedlings. Between 8 and 17 days after transplanting (DATP), leaf area of the first leaf was determined. In addition, 24 h after ^{14}C-sucrose application to the leaves, ^{14}C activity in plant parts other than the treated leaves was determined as translocated ^{14}C. Means and standard errors of leaf area (LA) and ^{14}C translocation (TR) are based on data from six replicates.

4.2.2. Absorption and translocation of ^{14}C-sucrose

A semi-quantitative description of the distribution of radioactivity from exogenous ^{14}C-sucrose was obtained by autoradiography. The first leaves of canola seedlings at the four-leaf stage were treated with chlorsulfuron. Twenty-four hours later, six droplets of solution containing ^{14}C-sucrose were applied to the treated leaves.

In control seedlings (*i.e.*, no chlorsulfuron treatment), ^{14}C-sucrose was translocated to all plant parts (Fig. 12, A). However, ^{14}C was distributed differently to the various plant parts. The images of treated spots and veins of ^{14}C-treated leaves were darker than those of the margins, indicating that ^{14}C was transported through the phloem. Most of the ^{14}C was translocated to the shoot apex (the third and fourth leaves) and the roots, the actively growing plant parts, while less ^{14}C was translocated to the second and cotyledonary leaves.

Chlorsulfuron-treated seedlings showed a difference from control seedlings. The third and fourth leaves and the roots were smaller in chlorsulfuron-treated seedlings than in control seedlings (Fig. 12, B). Regardless of the small size of the actively growing parts, chlorsulfuron-treated seedlings showed a distribution pattern of ^{14}C similar to that in control seedlings. However, the image of the ^{14}C-treated leaf of chlorsulfuron-treated seedlings was darker, suggesting that ^{14}C transport out of the leaf was reduced. The dark image in the first leaf and the small size of the third and fourth leaves of chlorsulfuron-treated seedlings suggest a relationship between ^{14}C transport reduction and growth reduction.

For a more precise quantitative assessment of sucrose translocation, component parts of similarly treated seedlings were combusted and their ^{14}C-content was determined. The first leaves of canola seedlings at the four-leaf stage were treated with chlorsulfuron. After 1, 6, 12 or 24 h, ^{14}C-sucrose was applied to the treated leaves (same-leaf treatment). Seedlings were harvested 24 h after ^{14}C-sucrose application. Eighty-seven percent of total applied radioactivity was recovered in both control and chlorsulfuron-treated plants.

Absorption of sucrose by leaves of control plants ranged between 12 and 16% of the

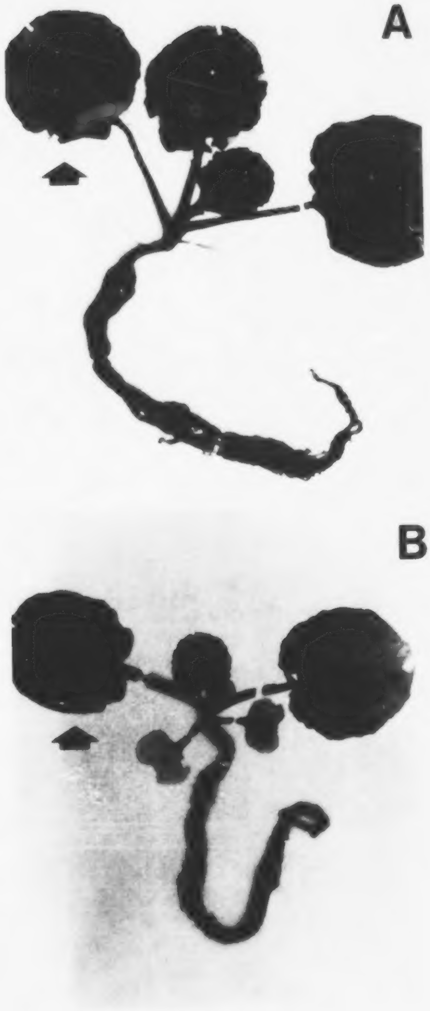

Figure 12. Distribution patterns of ^{14}C following ^{14}C-sucrose application to the first leaf (arrow marked) of control (A) and chlorsulfuron-treated (B) canola seedlings at the four-leaf stage. Twenty-four hours after chlorsulfuron application to the first leaf, ^{14}C-sucrose was applied to the same leaf. A further 24 h later, seedlings were harvested, dried, and autoradiographed. The dark images show strong ^{14}C activity whereas the light images indicate weak activity.

total applied radioactivity (Table 3). In chlorsulfuron-treated leaves, the data suggested a possible decrease in absorption 12 to 24 h after treatment. After 24 h, 75 to 84% of the recovered radioactivity in control plants was still in the treated leaf, and 16 to 25% had been exported to the rest of the plant, mainly to the shoot apex and the roots (5 to 8% each). In chlorsulfuron-treated plants, sucrose export from the treated leaf was unaffected for about 6 h, but then decreased to one half or less of that in control plants. These results for canola are consistent with reports of earlier experiments with *Thlaspi arvense* (Bestman *et al.* 1990a, Vanden Born *et al.* 1988) and *Arabidopsis thaliana* (Hall and Devine 1993).

In a further experiment, chlorsulfuron was applied to the first leaf of canola seedlings at the four-leaf stage, and ^{14}C-sucrose was applied to the second leaf (adjacent-leaf treatment). Sucrose was applied 1, 6, 12, or 24 h after chlorsulfuron treatment, and seedlings were harvested 24 h after sucrose application. Of the total applied radioactivity, 93% was recovered in both control and chlorsulfuron-treated plants.

Absorption of sucrose by leaves of control plants ranged between 10 and 15% of the total applied radioactivity, while that by the leaves of chlorsulfuron-treated plants ranged between 11 and 17% (Table 4). Control leaves absorbed similar amounts of ^{14}C in 24 h. The control leaves (the second leaves in Table 4) absorbed similar amounts of ^{14}C to the other control leaves (the first leaves in Table 3), indicating that fully expanded leaves absorbed quite similar amounts of sucrose regardless of leaf position and time after chlorsulfuron application.

In control plants, the data suggested a possible increase in translocation over time. After 24 h, 34% of the total recovered radioactivity had been exported to the rest of the plant, mainly to the shoot apex and the roots (8-14% each). The control leaves (the second leaves in Table 4) exported more sucrose than the other control leaves (the first leaves in Table 3), suggesting different assimilate-transporting abilities in those leaves. However, the nature of the differences in ability to transport sucrose between different leaves has not emerged from these experiments. In chlorsulfuron-treated plants, the leaves adjacent to chlorsulfuron-treated leaves exported similar amounts of sucrose to the shoot apex and roots 6 h after treatment. The leaves,

Table 3. Effect of chlorsulfuron on ^{14}C distribution following ^{14}C-sucrose application in canola seedlings (same-leaf treatment). Chlorsulfuron and ^{14}C-sucrose were applied to the first leaf of seedlings at the four-leaf stage. Means and standard errors (in parentheses) are based on data from three replicates.

Treatment	Time[1]	Absorption	Translocation	Treated leaf[2]	Adjacent leaf[3]	Shoot Apex[4]	Roots	Remainder[5]
	(h)	(% of applied)	(% of recovered)	(% of translocated)				
Control	1	16.3 (1.9)	24.9 (7.2)	75.1 (7.2)	4.1 (1.6)	8.0 (3.0)	9.1 (2.7)	3.8 (0.9)
	6	13.9 (0.5)	16.6 (1.4)	83.4 (1.4)	2.2 (0.7)	5.1 (0.6)	6.9 (0.4)	2.3 (0.1)
	12	15.2 (2.8)	15.8 (4.4)	84.2 (4.4)	2.1 (0.6)	5.6 (2.1)	6.3 (1.6)	1.8 (1.6)
	24	12.2 (0.7)	22.9 (1.8)	77.1 (1.8)	3.2 (0.7)	8.2 (0.8)	8.6 (1.0)	2.9 (0.4)
Chlorsulfuron	1	17.0 (2.1)	22.9 (8.2)	77.1 (8.2)	2.5 (0.9)	9.0 (3.8)	7.7 (2.3)	3.6 (1.9)
	6	13.6 (0.3)	16.8 (4.5)	83.1 (4.5)	3.1 (0.6)	5.2 (1.7)	5.9 (1.3)	2.7 (1.2)
	12	13.1 (2.0)	9.4 (2.0)	90.6 (2.0)	2.3 (0.9)	1.8 (0.6)	3.7 (0.6)	1.6 (0.7)
	24	8.7 (0.4)	9.6 (1.6)	90.4 (1.6)	2.7 (1.2)	2.7 (1.2)	2.9 (0.4)	1.2 (0.3)

[1] Interval of time between chlorsulfuron and ^{14}C-sucrose application.
[2] The first leaf.
[3] The second leaf.
[4] The third and fourth leaves.
[5] Stem and cotyledonary leaves.

Table 4 Effect of chlorsulfuron on ^{14}C distribution following ^{14}C-sucrose application in canola seedlings (adjacent-leaf treatment). Chlorsulfuron was applied to the first leaf and ^{14}C-sucrose to the second leaf of seedlings at the four-leaf stage. Means and standard errors (in parentheses) are based on data from three replicates.

Treatment	Time[1]	Absorption	Translocation	Treated leaf[2]	Adjacent leaf[3]	Shoot Apex[4]	Roots	Remainder[5]
	(h)	(% of applied)	(% of recovered)	(% of translocated)				
Control	1	12.5 (1.6)	23.3 (3.1)	76.7 (3.1)	2.7 (0.6)	9.2 (1.9)	8.1 (1.0)	3.2 (0.3)
	6	9.7 (0.4)	22.5 (7.7)	69.7 (0.5)	5.4 (3.7)	8.5 (1.6)	8.7 (4.3)	2.2 (0.1)
	12	12.4 (1.4)	30.5 (8.5)	69.5 (8.5)	2.4 (0.6)	12.0 (3.0)	12.7 (4.0)	3.3 (1.1)
	24	14.9 (1.1)	33.9 (2.3)	66.1 (2.3)	2.5 (0.9)	13.6 (1.2)	14.0 (1.2)	3.8 (0.5)
Chlorsulfuron	1	17.2 (2.0)	22.9 (8.6)	69.3 (5.9)	2.3 (0.8)	8.9 (0.5)	12.8 (1.0)	5.0 (0.9)
	6	12.7 (0.9)	23.7 (0.7)	76.3 (1.7)	4.1 (1.0)	8.3 (0.3)	7.8 (0.9)	3.5 (0.1)
	12	14.3 (2.4)	21.6 (5.4)	78.4 (5.4)	2.1 (0.9)	7.7 (2.4)	7.9 (0.9)	3.9 (1.3)
	24	11.3 (0.9)	25.7 (6.2)	74.3 (6.2)	2.9 (0.5)	6.6 (0.3)	8.6 (1.5)	3.3 (0.4)

[1] Interval of time between chlorsulfuron and ^{14}C-sucrose application.
[2] The second leaf.
[3] The first leaf.
[4] The third and fourth leaves.
[5] Stem and cotyledonary leaves.

however, exported less sucrose to the shoot apex and roots than the leaves in control plants during that period.

4.2.3. Exudation of sucrose by excised leaves

Researchers (King and Zeevaart 1974, Costello *et al.* 1982) have reported that EDTA, as a chelating agent, enhanced exudation of phloem-mobile metabolites. Therefore, a preliminary experiment was performed to determine the appropriate concentration of EDTA. The first leaves of canola seedlings at the four-leaf stage were excised and cut petiole ends were placed in phosphate buffer solution containing various concentrations of EDTA.

The optimal EDTA concentration for sugar exudation out of the excised leaves was 1 mM (Fig. 13). At 2.5 mM or higher concentrations, leaves exuded the same amount of sugar as without EDTA, possibly due to phytotoxicity. EDTA at 10 mM caused injury to the petioles, and the cut ends became twisted and hardened. On the basis of these results, 1 mM of EDTA was added to the buffer solution to maintain sugar exudation.

The first leaves of canola seedlings at the four-leaf stage were treated with chlorsulfuron as before. After 12, 24, or 48 h, the treated leaves were excised and cut ends were placed in phosphate buffer containing 1 mM of EDTA for 8 h. The exudates from excised leaves of both control and chlorsulfuron-treated seedlings contained sucrose and hexoses such as glucose and fructose. Forty-eight hours after treatment, excised leaves of control seedlings exuded 0.2 nmole of hexoses and 1.7 nmole of sucrose per leaf in 8 h. Excised leaves of chlorsulfuron-treated seedlings also exuded similar ratios of hexoses to sucrose. The amounts of hexoses, therefore, were combined with those of sucrose, since the hexoses are not the transporting sugar but the hydrolysates of sucrose (Delrot *et al.* 1983).

The excised leaves of control seedlings exuded a net amount of 1.1-2.0 nmoles of sucrose per mg FW during 8 h (Fig. 14). However, the amount of sucrose exuded in this experiment could be an under-estimation of the total amount of exudate because the exuded

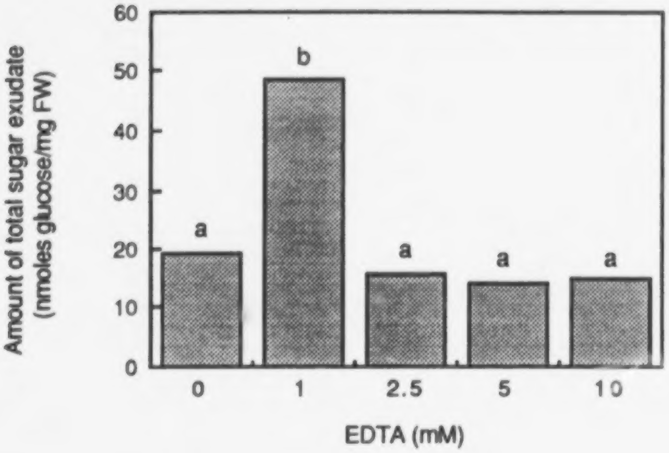

Figure 13. Effect of EDTA on sugar exudation in excised leaves of canola seedlings. Excised leaves were placed in phosphate buffer containing various concentrations of EDTA for 8 h. Values designated by the same letter are not significantly different by $LSD_{0.05}$ tests.

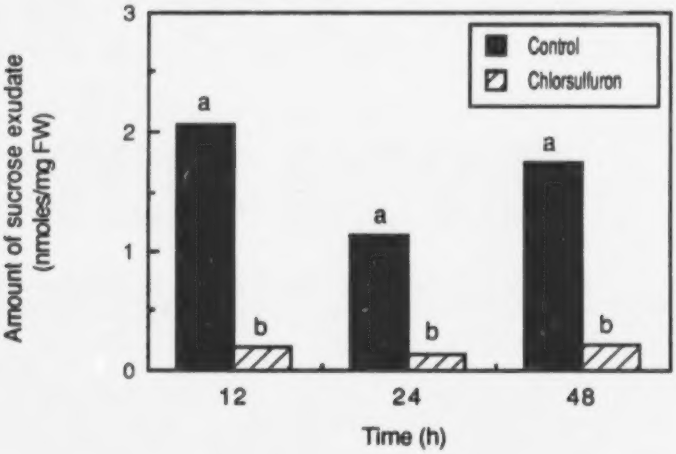

Figure 14. Exudation of sucrose out of excised leaves of canola seedlings. Excised leaves of canola seedlings were placed in phosphate buffer containing 1 mM EDTA for 8 h at 12, 24 and 48 h after chlorsulfuron treatment. Exudates contained sucrose and hexoses. The amounts of hexoses were combined with those of sucrose. Values designated by the same letter are not significantly different by $LSD_{0.05}$ tests.

sucrose could be translocated back to the leaves via transpiration. In another experiment, ^{14}C-sucrose applied to the buffer solution was found in the excised leaves, presumably via the transpiration stream (Appendix 2). Chlorsulfuron-treated excised leaves exuded less than 0.5 nmoles per mg FW in 8 h (Fig. 14), or 17 to 27% of the amounts for corresponding control leaves, regardless of the time elapsed since the chlorsulfuron treatment. These results are consistent with the findings of Bestman *et al.* (1990a), for leaves of *Thlaspi arvense*.

4.2.4. Discussion

Data from a quantitative assessment of ^{14}C-sucrose translocation showed that when chlorsulfuron-treated leaves of canola seedlings were treated with ^{14}C-sucrose (same-leaf treatment), the treated leaves showed decreased ^{14}C transport 12 h after chlorsulfuron treatment (Table 3). Similar results were observed in an autoradiography study. Although the autoradiogram of chlorsulfuron-treated canola seedlings was similar to that of control seedlings, two significant differences were found. Firstly, the margins of ^{14}C-treated leaves of chlorsulfuron-treated seedlings were darker than those of control seedlings. Secondly, the actively growing parts of chlorsulfuron-treated seedlings were smaller in size than those of control seedlings. The former result suggests that chlorsulfuron reduced ^{14}C export out of a treated leaf, while the latter suggests that chlorsulfuron reduced such export at least in part by inhibiting the growth of the actively growing plant parts. Although these results suggest that chlorsulfuron reduced ^{14}C export out of a treated leaf, they raise a difficult question on the action site of chlorsulfuron. If the reduced ^{14}C export is due to herbicide effects in the treated leaves, the main action site of chlorsulfuron would be in the treated leaves. In this case, the small size of actively growing parts of chlorsulfuron-treated canola seedlings has resulted from the reduction in assimilate translocation. On the other hand, if the reduced ^{14}C export is due to herbicide effects in the actively growing parts, the main action site of chlorsulfuron would be in the actively growing parts.

Chlorsulfuron-treated excised canola leaves showed reduced exudation of sucrose, the major transporting carbohydrate in the plants, indicating that chlorsulfuron reduced sucrose export out of the treated leaves. Similar results were reported by Bestman *et al.* (1990a) and Lowther (1990) for another species. These results support the view that the main action site of chlorsulfuron is in the treated leaves. The dysfunction of membranes (Vanden Born *et al.* 1988) and of proteins involved in sucrose transport into the phloem (Bestman *et al.* 1990a, Lowther 1990) has been suggested as a putative inhibitory mechanism. However, the finding that leaf discs of both control and chlorsulfuron-treated canola leaves showed the same degree of electrolyte leakage suggests that effects of chlorsulfuron on sucrose export might not be due to the dysfunction of membranes (Appendix 4). A recent study by Hall and Devine (1993) showed that the reduction of assimilate export was not due to effects on H^+-ATPase that plays a pivotal role in phloem loading of assimilates in the plasma membrane. The scenario for chlorsulfuron-induced malfunctioning of proteins might be as follows: chlorsulfuron inhibits the acetolactate synthase enzyme, the primary target. The interaction of chlorsulfuron with acetolactate synthase results in a shortage of branched-chain amino acids, namely, leucine, valine, and isoleucine. This blocks synthesis of proteins that are involved in sucrose transport into the phloem. However, this scenario *per se* is an arguable point. Some researchers (Brunk and Rhodes 1988, Rhodes *et al.* 1987) reported that chlorsulfuron-treated plants did not deplete pools of branched-chain amino acids by proteolysis. If chlorsulfuron induces proteolysis when chlorsulfuron-treated leaves have reduced assimilate export, then proteins such as Rubisco that represents a large protein pool are expected to be recycled to synthesize the essential proteins including those which are involved in sucrose transport into the phloem. Therefore, the proteins involved in sucrose transport into the phloem are expected to maintain their functions.

The suggestion that chlorsulfuron inhibits functions of membranes or of proteins which, in turn, reduces assimilate export, is based on the conclusion that *Thlaspi* (Bestman *et al.* 1990a, Lowther 1990) and *Arabidopsis* (Hall and Devine 1993) plants load assimilate apoplastically. In this study, leaf explants ceased ^{14}C-sucrose uptake and translocation in the presence of PCMBS (Appendix 5), suggesting that canola plants also load assimilate apoplastically. Some plants, such as *Cucumis* (Schmitz *et al.* 1987), *Ipomoea* (Madore and Lucas 1987), and *Coleus* (Turgeon and Gowan 1990), load assimilate symplastically. Anatomically, the mesophyll cells and the sieve-element-and-companion-cells of these plants are connected by plasmodesmata. Since these symplastic-phloem-loading plants do not have the membrane proteins that are involved in sucrose transport into the phloem as apoplastic-phloem-loading plants have, the absence or presence of a herbicide effect on reduction of assimilate transport in symplastic-phloem-loading plants will help to understand whether the reduced assimilate transport in *Thlaspi arvense* (Bestman *et al.* 1990a), *Arabidopsis* (Hall and Devine 1993), Tartary buckwheat (Vanden Born *et al.* 1988), and canola is due to a malfunction of proteins in the plasma membrane.

In chlorsulfuron-treated canola seedlings, a reduction in sucrose transport out of chlorsulfuron-treated leaves can result in a starvation for carbohydrate in actively growing plant parts. Carbohydrate starvation is known to induce a number of metabolic and physiological changes such as reductions in respiration (Elamrani *et al.* 1994, Brouquisse *et al.* 1991, Journet *et al.* 1986) and cell division (Kodama *et al.* 1994, Gould *et al.* 1981), loss in greening capacity (Elamrani *et al.* 1994), and an increase in proteolysis (James *et al.* 1993). Some of these changes, for example cell division (Ray 1984, Rost and Reynolds 1985) and proteolysis (Brunk and Rhodes 1988, Rhodes *et al.* 1987), occurred also in chlorsulfuron-treated plants. Therefore, the actively growing parts of chlorsulfuron-treated canola seedlings which are starved for carbohydrate might induce those metabolic and physiological changes which, in turn, result in growth reduction. This again suggests that the main action site of chlorsulfuron is in the treated leaves, and the growth reduction in the actively growing parts is, in part, a consequence of the reduction in assimilate translocation.

Data from adjacent-leaf experiments, however, support the view that the main growth-inhibition action site of chlorsulfuron is in the actively growing plant parts. In chlorsulfuron-treated plants, the leaves adjacent to chlorsulfuron-treated leaves exported similar amounts of sucrose to the shoot apex and roots 6 h after treatment, while in control plants, the same leaves exported more sucrose to the shoot apex and roots than the leaves of the plants after 6 h (Table 4). A herbicide effect in the shoot apex and the roots accounts for the absence of an increase in sucrose export.

In conclusion, chlorsulfuron reduced transport of both exogenous ^{14}C-sucrose and endogenous sucrose out of treated leaves of canola seedlings. The action site of chlorsulfuron was in two different parts of the treated seedlings; both in the treated leaf and in the actively growing plant parts. In treated leaves, chlorsulfuron reduced export of assimilates. However, the precise nature of chlorsulfuron action on the reduced export of assimilates has not become clear in this research. In the actively growing plant parts, chlorsulfuron that was translocated there from the treated leaf resulted in reduced growth which, in turn, reduced assimilate export. The overall data indicate that, in chlorsulfuron-treated canola seedlings, a herbicide effect on reduced assimilate translocation is due to the disruption of a source-sink relationship.

4.3. Effect of chlorsulfuron on carbohydrate production and allocation

The aim of the series of experiments in this section was to examine whether the reduced sucrose translocation in chlorsulfuron-treated canola seedlings was due to reduced carbohydrate production or to changed carbon allocation. Experiments on chlorophyll content, photosynthesis, transpiration, carbohydrate contents, and sucrose phosphate synthase activity were conducted to examine the effect of chlorsulfuron on carbohydrate production. Allocation of carbon was studied by examining respiration, amino acid content, and activities of sucrose phosphate synthase (SPS), phosphoenol pyruvate carboxylase (PEPcase), and nitrate reductase (NR).

4.3.1. Chlorophyll content

The first leaves of canola seedlings at the four-leaf stage were treated with chlorsulfuron. Chlorophyll content was measured to determine whether chlorsulfuron treatment affected the light-harvesting pigments.

Chlorophyll content in control leaves remained nearly constant during the 96-h experiments, while that in chlorsulfuron-treated leaves gradually fell to about 65% of the control level (Fig. 15). The decrease became noticeable between 24 and 48 h after chlorsulfuron treatment. However, the treated leaves did not show chlorosis (Fig. 6 in section 4.1.2), the typical symptom of chlorophyll-synthesis inhibitors.

4.3.2. Photosynthesis

The first leaves of canola seedlings at the four-leaf stage were treated with chlorsulfuron. Twenty-four hours after treatment, the leaves were excised and net carbon exchange (NCE) rates were measured.

Chlorsulfuron treatment had no measurable effect on NCE rates (Fig. 16, A). In both

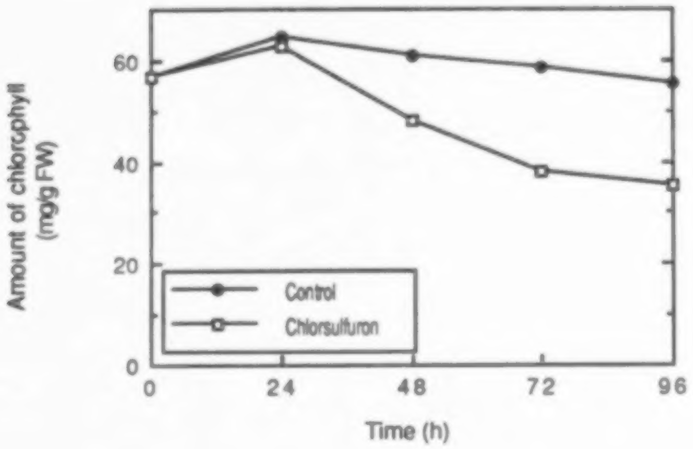

Figure 15. Effect of chlorsulfuron on chlorophyll content in leaf discs from the first leaves of canola seedlings. *F*-values for the main effects of chlorsulfuron and time and their interaction were all significant at the $P_{0.01}$ level.

control and chlorsulfuron-treated excised leaves, the NCE rates increased rapidly during the first 75 min after leaf excision (Fig. 16, B). Thereafter, the NCE rates decreased slowly over the 8-hour experimental period. The results indicate that chlorsulfuron did not have an effect on the NCE rates in the treated canola leaves.

4.3.3. Transpiration

Transpiration rates were measured simultaneously with NCE rates. Patterns of transpiration of control and chlorsulfuron-treated excised leaves (Fig. 17) were similar to those of the NCE rates (Fig. 16). Initially, they increased rapidly and reached a maximum level 75 min after leaf excision (data not shown; the pattern of change was the same as in Fig. 16, B). The maximum transpiration rate in both control and chlorsulfuron-treated leaves (1.2 mmol m^{-2} s^{-1}) remained nearly constant for 4 hours, and then decreased.

4.3.4. Respiration

The first leaves of canola seedlings at the four-leaf stage were excised 24 h after chlorsulfuron treatment. The leaves were incubated in darkness, and the respiration rate was determined by measuring NCE.

Twenty-four hours after treatment, control and chlorsulfuron-treated leaves showed similar NCE rates at the initial period of measurement (Fig. 18). In control leaves, the NCE rate decreased over time, with oscillation (average slope = - 0.96 µmol m^{-2} s^{-1}). The respiration was lowest at the end of the experiment (- 0.14 µmol m^{-2} s^{-1}).

Chlorsulfuron-treated leaves showed a different pattern of respiration. The NCE rate remained constant. Hence, respiration of treated leaves was significantly greater than that of control leaves ($P < 0.01$).

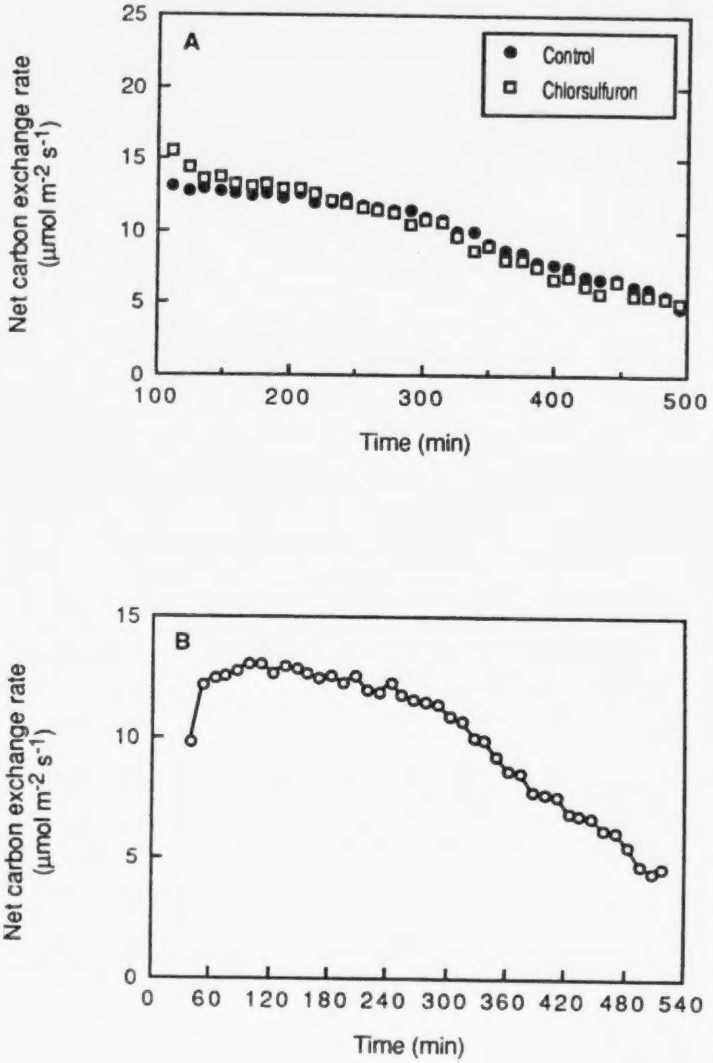

Figure 16. Net carbon exchange rate of excised leaves of chlorsulfuron-treated canola seedlings. The leaves were exposed to 350 μl l^{-1} CO_2. Data were collected every 12 min for 8 h. The data plotted are based on the results of three replicates. A, the NCE rates of both control and chlorsulfuron-treated leaves between 100 and 500 min after leaf excision; B, the NCE rates of control leaves for 8 h.

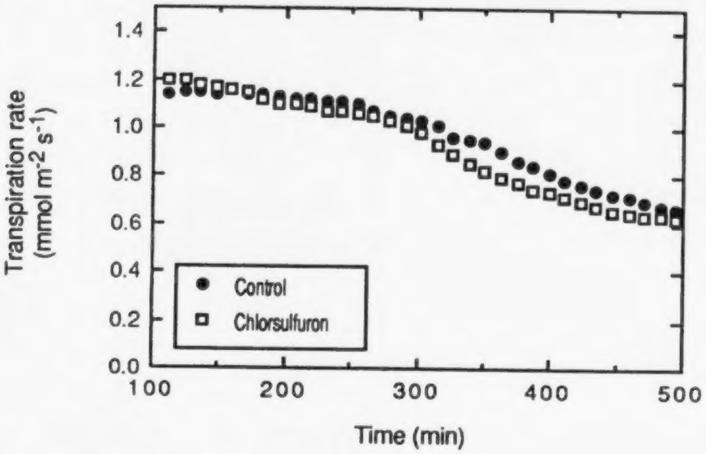

Figure 17. Transpiration rate of excised leaves of chlorsulfuron-treated canola leaves. The leaves were exposed to 350 µl l^{-1} CO_2. Data were collected every 12 min for 8 h. Data plotted are based on results of three replicates.

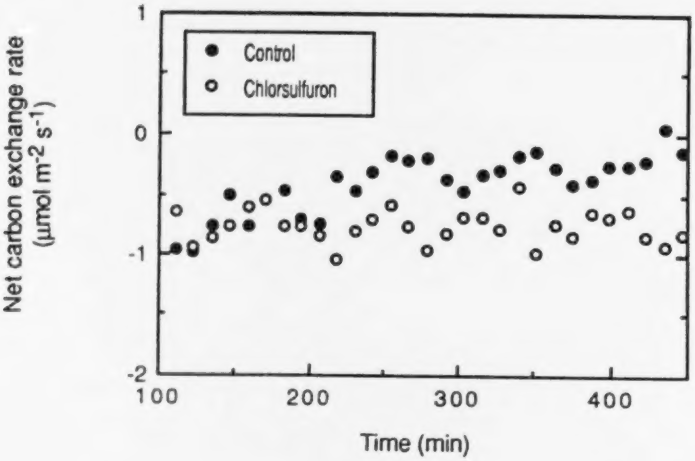

Figure 18. Respiratory CO_2 efflux rate of excised leaves of chlorsulfuron-treated canola seedlings. The leaves were exposed to 350 μl l^{-1} CO_2 under darkness. Data were collected every 12 min for 8 h. The data plotted are based on results of three replicates. *F*-values for the main effects of chlorsulfuron and time and their interaction were all significant at the $P_{0.01}$ level. Negative values indicate that efflux of CO_2 from leaves is higher than influx.

4.3.5. Sugar content

The first leaves of canola seedlings at the four-leaf stage were treated with chlorsulfuron, and the sugar content was measured after 12, 24, or 48 h.

In control leaves, contents of fructose, glucose, and total sugar were relatively constant for 48 h (Table 5). Although glucose content appeared to increase between 24 and 48 h, the change was not significant. The leaves had higher amounts of sucrose at 48 h than at 24 h, indicating that the leaves at that time produced more sucrose than any other sugars.

Chlorsulfuron-treated leaves contained similar amounts of fructose, glucose, sucrose, and total sugar to control leaves 24 h after treatment (Table 5). However, 48 h after treatment, the contents of glucose, sucrose, and total sugar in the treated leaves were greater than those at 24 h. The results indicate that chlorsulfuron caused an accumulation of free sugars 24 h after treatment.

4.3.6. Starch content

The first leaves of canola seedlings at the four-leaf stage were treated with chlorsulfuron. Starch content was measured as starch-derived glucose to determine whether chlorsulfuron treatment altered carbohydrate partitioning to starch.

In control leaves, the starch content remained relatively constant during the experiment (Fig. 19). Data show a decrease between 12 and 24 h; however, the starch contents were not significantly different ($P = 0.1$).

In chlorsulfuron-treated leaves, the starch content was similar to that in control leaves between 12 and 24 h after treatment. Forty-eight hours after treatment, however, the content in the treated leaves was higher than that in control leaves, indicating that chlorsulfuron caused an accumulation of starch 24 h after treatment.

Table 5. Total extractable sugars in chlorsulfuron-treated leaves of canola seedlings. Data are based on the results from six replicates.

Time (h)	Sugars Control		Sugars Chlorsulfuron		Herbicide Effect[1] (% of control)	
	Fructose[2] (nmoles/mg FW)					
12	1.10	a	0.94	a	86	
24	1.46	a	1.65	a	113	
48	1.10	a	1.51	a	137	
	Glucose (nmoles/mg FW)					
12	0.88	a	0.81	a	92	
24	0.79	a	1.17	a	148	
48	1.33	a	2.54	b	191	**
	Sucrose (nmoles/mg FW)					
12	0.96	a	0.81	a	84	
24	0.75	a	0.82	a	109	
48	1.87	b	2.39	b	128	
	Total Sugar[3] (nmoles glucose/mg FW)					
12	43.6	a	36.8	a	85	
24	47.7	a	43.7	a	92	
48	46.7	a	71.5	b	153	**

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[1] Significance (rows) at $P < 0.01$ (**).

[2] Values designated by the same letter (columns in each sugars) are not significantly different by $LSD_{0.05}$ tests.

[3] Total neutral hexoses including galactose, mannose, glucose, fucose (Spiro 1966).

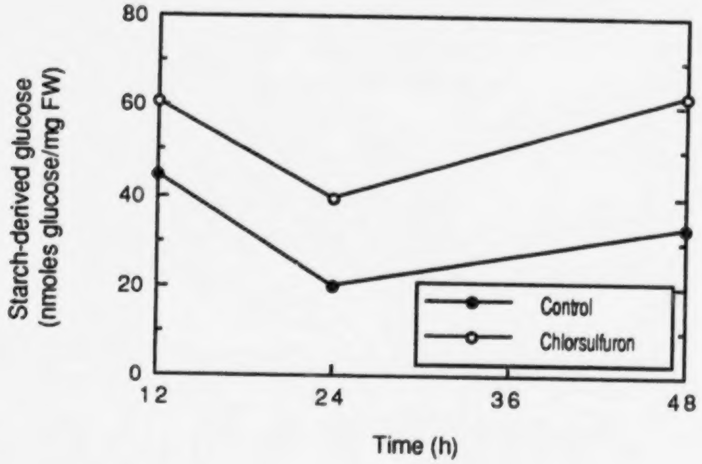

Figure 19. Effect of chlorsulfuron on starch content, measured as starch-derived glucose, in control and chlorsulfuron-treated leaves of canola seedlings. Data plotted are based on results of six replicates. *F*-value for the main effects of chlorsulfuron and time and their interaction were significant at the $P_{0.01}$, $P_{0.06}$ and $P_{0.76}$ levels, respectively.

4.3.7. Free amino acid content

The first leaves of canola seedlings at the four-leaf stage were treated with chlorsulfuron, and the free amino acid content in the seedlings was measured. The free amino acids measured in this experiment reflect a mixture of amino acids derived from *de novo* synthesis and from proteolysis (Singh and Shaner 1995).

In control leaves, the amino acid content was 23.6 nmoles mg^{-1} FW 12 h after treatment and increased slightly 24 h after treatment (Fig. 20). Thereafter, it decreased and showed a much lower level than at 12 h after treatment. The decrease in free amino acids 24 h after treatment could be due to a dilution factor by leaf growth.

Chlorsulfuron-treated leaves showed a similar pattern in free amino acid content; however, the treated leaves had a higher free amino acid content than control leaves (Fig. 20). Twelve hours after treatment, the amino acid content in the treated leaves was similar to that in control leaves. Thereafter, the treated leaves had 78% and 253% higher free amino acid content 24 and 48 h, respectively, after treatment than the control leaves.

4.3.8. Protein

The aim of this experiment was to examine whether chlorsulfuron treatment resulted in protein hydrolysis which could contribute to the increase in free amino acids. The first leaves of canola seedlings at the four-leaf stage were treated with chlorsulfuron, and the protein content and protein profiles in the treated leaves were determined.

In control leaves, the protein content at 0 h was 8.69 µg mg^{-1} FW (Table 6). It was relatively constant for at least 24 h. In chlorsulfuron-treated leaves, protein content changed in a similar manner as in control leaves, suggesting that chlorsulfuron did not affect the protein content.

The composition patterns of protein taken from control and chlorsulfuron-treated leaves

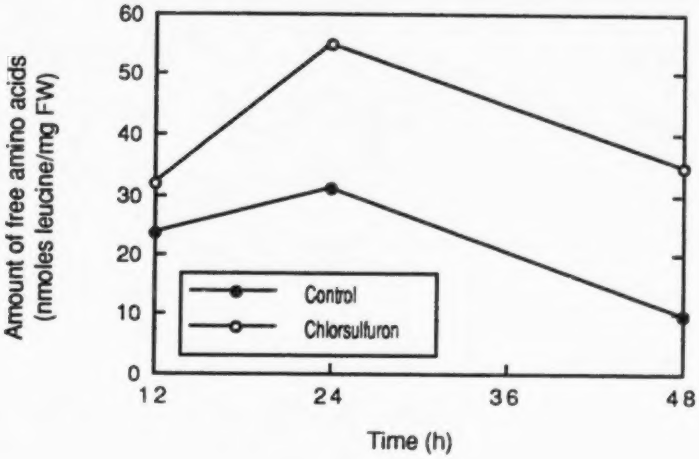

Figure 20. Free amino acid content of excised leaves of chlorsulfuron-treated canola seedlings. The amounts of leucine equivalent amino acid in the leaves were measured. The data plotted are based on results of six replicates. *F*-values for the main effects of chlorsulfuron and time and their interaction were significant at the $P_{0.01}$, $P_{0.01}$ and $P_{0.05}$ levels, respectively.

Table 6. Protein content of chlorsulfuron-treated leaves of canola seedlings at the four-leaf stage. Data are based on results from three replicates.

Time	Control		Chlorsulfuron		Herbicide Effect[1]	
(h)	(μg protein/mg FW[2])				(% of control)	
0	8.69	a				
12	8.64	a	9.75	a	113	ns
24	8.63	a	8.78	a	102	ns
48	10.68	ab	12.52	b	117	ns

[1] No significance (in rows) at $P = 0.05$.

[2] Fresh weight.

[3] Values designated by the same letter (in columns) are not significantly different at $P = 0.05$.

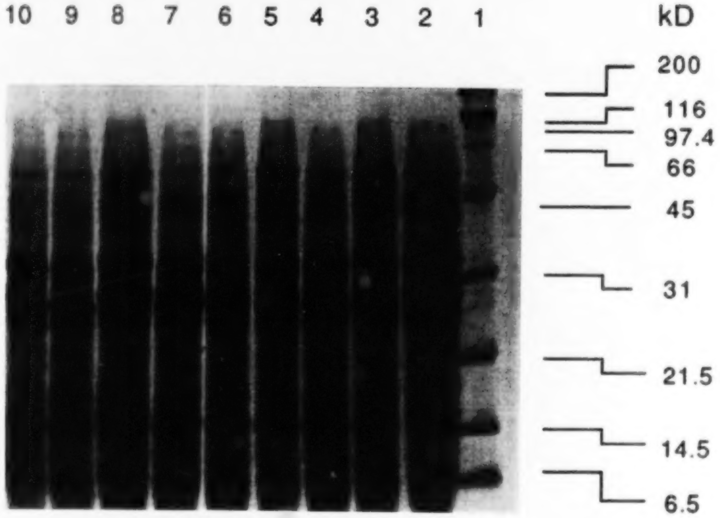
10
9
8
7
6
5
4
3
2
1
kD
200
116
97.4
66
45
31
21.5
14.5
6.5

Figure 21. Profiles of total soluble proteins extracted from chlorsulfuron-treated leaves of canola seedlings. Proteins were extracted, separated by SDS-PAGE, and Coomassie stained. Lane 1, molecular marker; 2,3,4,5, and 6 are profiles from control leaves at 0, 3, 6, 12, and 24 h, respectively. Lanes 7, 8, 9, and 10 are profiles from chlorsulfuron-treated leaves 3, 6, 12, and 24 h after treatment.

were similar in SDS-PAGE (Fig. 21). There were numerous protein bands, therefore it was not valid to draw any conclusion as to whether chlorsulfuron-treated leaves showed disappearance of protein bands by proteolysis.

4.3.9. Enzyme activity

The first leaves of canola seedlings at the four-leaf stage were treated with chlorsulfuron, and the activities of enzymes that are related to sucrose synthesis (phosphoenol pyruvate carboxylase and sucrose phosphate synthase) and amino acid synthesis (nitrate reductase) were measured.

4.3.9.1. Sucrose phosphate synthase

Sucrose phosphate synthase (SPS) catalyzes the conversion of UDPglucose and fructose-6-phosphate to sucrose-6-phosphate and UDP (uridine diphosphate). The SPS activity is maximized under Vmax conditions (without Pi, high substrate) and minimized under limiting conditions (with Pi, low substrate) (Huber and Huber 1992). The SPS activity, therefore, was measured under both types of conditions.

Under Vmax conditions, SPS activity of control leaves was highest (4.1 μmol h^{-1} g^{-1} FW) at 3 h (Fig. 22). Thereafter no substantial change was found due to high variation in the activity. Although data showed a diurnal pattern in the SPS activity (low activity in the morning and high activity at noon), no conclusion was drawn due to lack of over 24-h monitoring data.

Under limiting conditions, SPS activity of both control and chlorsulfuron-treated leaves was much lower than under Vmax conditions (Fig. 22). The SPS showed steady state activity (0.3-0.5 μmol h^{-1} g^{-1} FW), suggesting that the enzyme activity was limited by high amounts of Pi (inhibitor) and low amounts of fructose-6-phosphate (substrate).

In chlorsulfuron-treated leaves, SPS activity was the same as that in control leaves

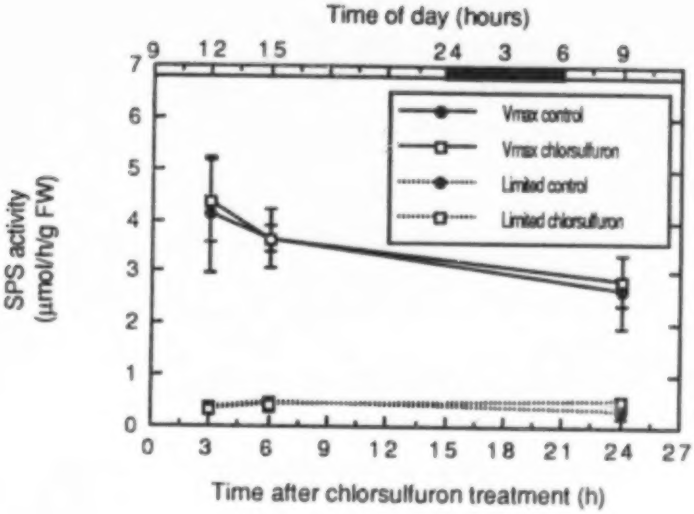

Figure 22. Effect of chlorsulfuron on activity of sucrose phosphate synthase. The enzyme activity of control and chlorsulfuron-treated leaves of canola seedlings was determined under Vmax (without Pi, high substrate) and limiting condition (with Pi, high substrate). Means and standard errors are based on data from five replicates. The shaded bar on top of figure indicates periods of darkness.

under both types of conditions (Fig. 22), indicating that chlorsulfuron did not affect SPS activity.

4.3.9.2. Phosphoenol pyruvate carboxylase

Phosphoenol pyruvate carboxylase (PEPcase) catalyzes the conversion of phosphoenol pyruvate to malate in the cytoplasm, and activation of PEPcase changes carbon flow to organic acids and amino acids (Champigny and Foyer 1992).

In control leaves, the Michaelis-Menten constant (Km) and the maximum rate of reaction (Vmax) of PEPcase were 1.03 mM PEP and 3.7 μmol PEP min^{-1} g^{-1} FW, respectively (Fig. 23, A). The PEPcase activity was highest at 3 h (7.4 μmol min^{-1} g^{-1} FW) (Fig. 23, B). Thereafter no substantial change was found.

In chlorsulfuron-treated leaves, the PEPcase activities were similar to those in control leaves (Fig. 23, B), suggesting that chlorsulfuron did not affect PEPcase.

4.3.9.3. Nitrate reductase

Nitrate reductase (NR) catalyzes the assimilation of nitrate to nitrite in the cytoplasm (Li and Oaks 1993). The assimilation of nitrate to glutamate-series amino acids requires not only energy but also carbon backbones. This process, therefore, competes for carbohydrate with the sucrose synthesis process (Rufty *et al.* 1992, Foyer *et al.* 1994).

Activity of NR was measured *in vivo*. In control leaves, the activity of NR was higher during the day than the night (Fig. 24, A). The enzyme activity was 24 nmoles KNO_2 g FW^{-1} h^{-1} at 9 am (at 0 h). Thereafter it increased in the early afternoon (at 3 h), and then decreased to a minimum at 3 am (at 18 h). The activity increased again, indicating a diurnal rhythm.

In chlorsulfuron-treated leaves, the diurnal pattern also occurred but after 12 h the activity in the leaves gradually increased to a level nearly double that in controls. A similar result was obtained in a repeated experiment. Twenty-four hours after treatment, the NR of the treated

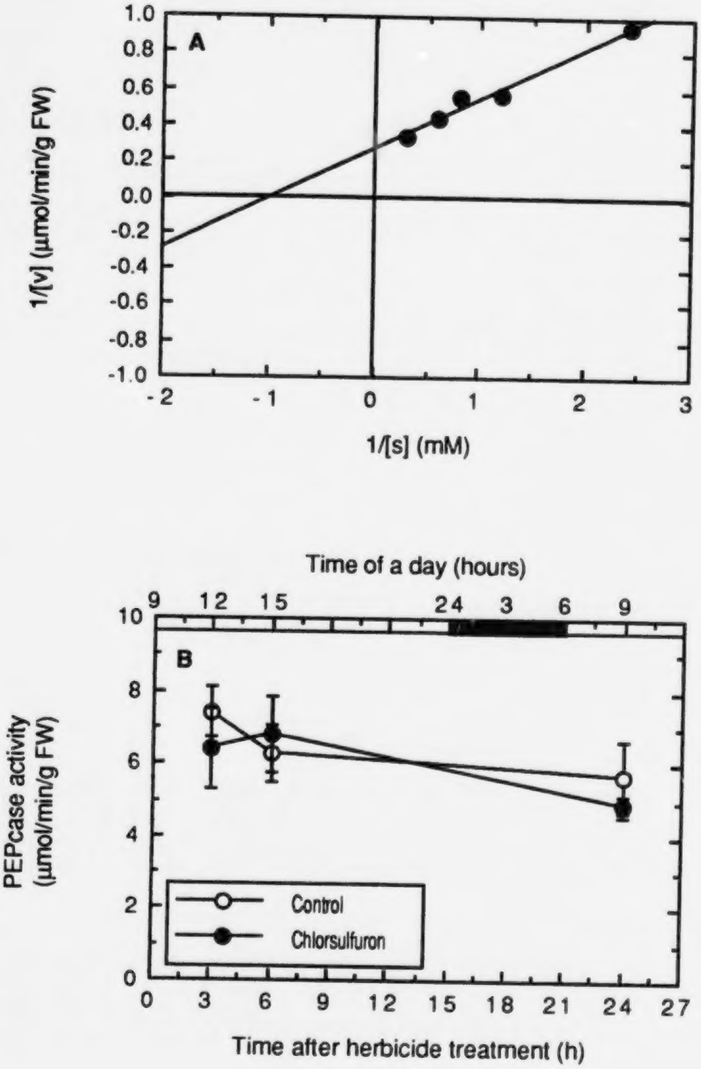

Figure 23. Phosphoenol pyruvate carboxylase activity of chlorsulfuron-treated canola leaves. A, double reciprocal Lineweaver-Burk plot for PEPcase in control leaves; B, PEPcase activity over time. Means and standard errors are based on data from five replicates. The shaded bar on top of B indicates periods of darkness.

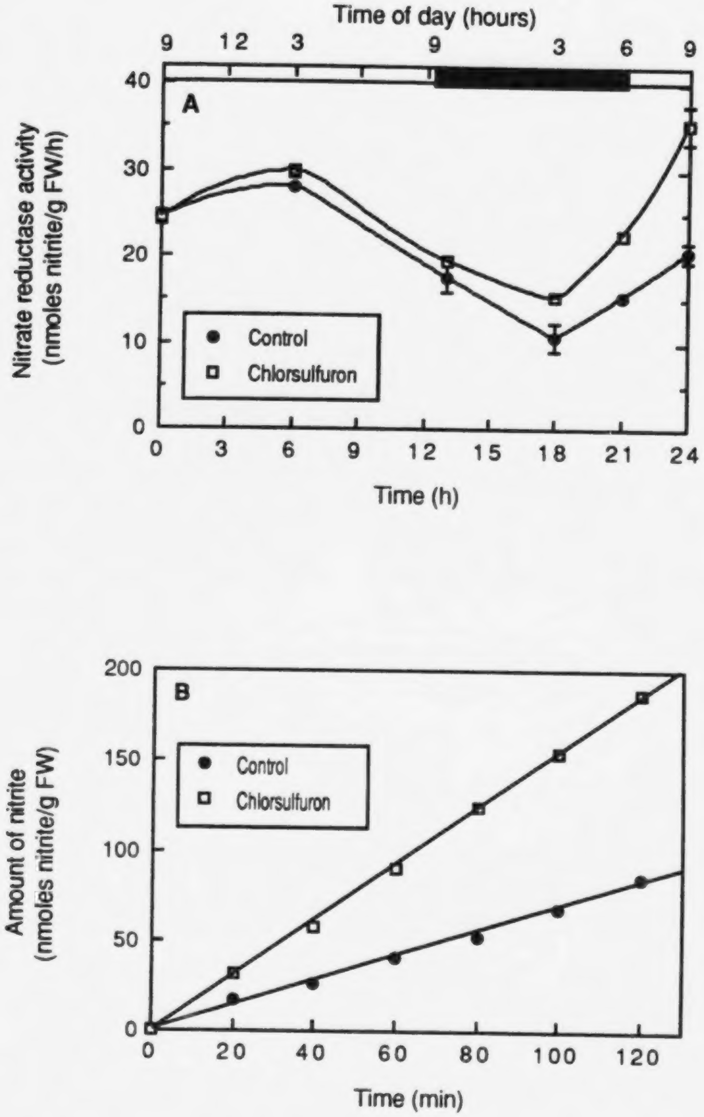

Figure 24. Effect of chlorsulfuron on nitrate reductase activity in leaf discs from the first leaves of canola seedlings. The leaf discs were incubated in phosphate buffer solution containing nitrate. A, nitrate reductase activity during 24 h after chlorsulfuron treatment. *F*-values for the main effects of chlorsulfuron and time and their interaction were significant at the $P_{0.01}$, $P_{0.01}$ and $P_{0.05}$ levels, respectively; B, nitrite production by leaf discs from chlorsulfuron-treated canola leaves between 24 and 26 h after treatment. Means are based on results from three replicates.

leaves increased nitrite amount linearly at the rate of 1.56 nmoles g^{-1} FW, while that of control leaves increased at the rate of 0.69 nmoles g^{-1} FW (Fig. 24, B).

4.3.10. Discussion

The first leaves of canola seedlings treated with 1 µg of chlorsulfuron showed marked concentration changes in some carbohydrates 48 h after treatment (Table 7). In addition to changes in carbon metabolism, such leaves exported less sucrose than control leaves during that period (Table 3, Fig. 14). However, the change in sucrose transport (12 h) occurred much earlier than the changes in carbohydrate status. In order to understand why chlorsulfuron-treated plant leaves reduce assimilate transport, metabolic and physiological changes that occurred before the changes in carbohydrate status (24 h after chlorsulfuron treatment) were the focus in this research. However, metabolic and physiological changes that occurred 48 h after chlorsulfuron treatment in treated canola leaves are discussed with an emphasis on the cause of death in chlorsulfuron-treated plants.

The fact that chlorsulfuron-treated canola leaves had more starch and other soluble sugars than control leaves at 48 h confirms the previous findings of other researchers (Bestman *et al.* 1990a, Chao *et al.* 1993). In treated canola leaves, however, it was surprising not to detect an accumulation of sucrose which was reported for chlorsulfuron-treated *Thlaspi arvense* (Bestman *et al.* 1990a, Lowther 1990). The results might reflect different carbohydrate metabolism patterns that sequester carbon in different species. Since chlorsulfuron-treated canola leaves had higher amounts of glucose and hexoses (total sugar) than of sucrose, sucrose that was not exported probably was stored as hexoses after hydrolysis.

Chlorophyll content of chlorsulfuron-treated leaves 48 hours after treatment was lower than that of control leaves. However, the nature of the linkage between chlorsulfuron and chlorophyll content is not known.

The reduction in sucrose transport from source leaves of chlorsulfuron-treated canola

Table 7. Summary of chlorsulfuron effects on production and allocation of carbohydrate in chlorsulfuron-treated excised leaves and chlorsulfuron-treated leaves of intact canola seedlings at the four-leaf stage.

Physiological and metabolic processes	Effect of chlorsulfuron[*] At 24 h	after 24 h	Source
Chlorophyll content	No change	Decrease (96 h)	Fig. 15
Net carbon exchange rate	No change	-	Fig. 16
Transpiration	No change	-	Fig. 17
Respiration	No change (but increase during 8-h experiments)	-	Fig. 18
Sugar content			Table 4
Fructose	No change	No change (48 h)	
Glucose	No change	2-fold increase (48 h)	
Sucrose	No change	No change (48 h)	
Total sugar	No change	1.5-fold increase (48 h)	
Starch content	No change	Increase (48 h)	Fig. 19
SPS activity	No change	-	Fig. 22
PEPcase activity	No change	-	Fig. 23
Total amino acid content	2-fold increase	5-fold increase (48 h)	Fig. 20
NR activity	2-fold increase	-	Fig. 24
Protein content	No change	No change (48 h)	Table 5
Protein profile	?	-	Fig. 21

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[*] Comparison between control and chlorsulfuron-treated leaves. The change does not reflect the pattern over time.

seedlings could possibly result in a shortage of carbohydrate in sink parts of the seedlings. Sinks rely for their carbohydrate supply on the source leaves. Therefore, a reduction of carbohydrate supply from the source leaves inhibits sink growth. Carbohydrate starvation is known to induce a number of metabolic and physiological changes such as reduction in respiration and cell division, loss in greening capacity, and an increase in proteolysis (see sections 4.2.4 and 4.2.5). In conjunction with herbicidal effects of chlorsulfuron on branched-chain-amino-acid and pantothenic acid biosynthesis in sinks, those metabolic and physiological changes induced by chlorsulfuron could reduce sink growth and lead to the death of chlorsulfuron-treated plants.

The NCE and transpiration rates in chlorsulfuron-treated canola excised leaves were not different from those in control leaves, suggesting that chlorsulfuron did not inhibit photosynthesis processes in the treated canola leaves. The NCE rate in both chlorsulfuron-treated and control canola leaves decreased curvilinearly 4 h after reaching a maximum (Fig. 16). The conclusions reached confirm earlier results (Hatzios and Howe 1982, de Villiers *et al.* 1980, Vanden Born *et al.* 1988). Ray (1980) reported, for example, that chlorsulfuron up to 100 ppm (the same herbicide concentration as used in this research) had no effect on CO_2 fixation by isolated spinach leaf cells for 2 hours.

Twenty-four hours after chlorsulfuron treatment, an absence of herbicide effects on photosynthesis in treated canola leaves might be associated with carbohydrate contents and sucrose-synthesis-related enzyme activity. The treated leaves had similar levels of glucose, fructose, sucrose, total sugar, and starch to control leaves (Table 5, Fig. 19). In addition, the treated leaves had similar activities of SPS, one of the most important enzymes in sucrose synthesis. These results suggest that chlorsulfuron did not inhibit carbohydrate production. They imply, also, that the reduction in sucrose export out of chlorsulfuron-treated canola leaves was, at least, not due to a shortage of sucrose, the transporting carbohydrate (Fig. 14).

Twenty-four hours after treatment, the respiration rate in chlorsulfuron-treated leaves was not different from that in control leaves. Respiration is dependent on substrate status in plants (Azcon-Bieto and Osmond 1983, Musgrave *et al.* 1986). Hence, the similarities in pattern of respiration between control and treated leaves after 24 h could be related to the similar carbohydrate status in those leaves.

Accumulation of 2-ketobutyrate or 2-aminobutyrate in chlorsulfuron-treated plants has been suggested to disturb many metabolic and physiological processes such as cell division (Lanzagorta *et al.* 1988, Reid *et al.* 1985) and eventually to kill plants (LaRossa and Van Dyk 1987, LaRossa 1987, Schloss 1989). The final target of 2-ketobutyrate as a toxicant is suggested to be a component of the complex PEP-dependent phosphotransferase system which results in a decline in concentration of glycolytic intermediates either upstream (G6P, F6P, F1,6BP) or downstream (acetyl CoA) from PEP (Danchin *et al.* 1984). If this suggestion is correct, chlorsulfuron-treated canola leaves should have shown a reduction in respiration because of a shortage of precursors for respiration. However, results from respiration in chlorsulfuron-treated canola leaves did not show the expected reduction in respiration. The earlier results with selected metabolites in 2-ketobutyrate-treated seedlings (Fig. 9 and 10) support the conclusion that effects of those toxic intermediates were not involved in the herbicide effect of chlorsulfuron in canola seedlings.

Chlorsulfuron-treated canola leaves showed a time gap between the reduction in sucrose transport (12 h after treatment) and the increase in carbohydrate content (48 h after treatment). During that period, chlorsulfuron-treated canola leaves had a similar level of carbohydrates to control leaves, while the treated leaves had a higher respiration rate than control leaves. These results suggest that the treated leaves might change carbon allocation to other plant constituents during that period and, as a consequence, the leaves reduced the sucrose transport. In chlorsulfuron-treated canola leaves, the plant constituents or physiological processes that showed changes within 48 h after treatment were the free amino acids and respiration. The treated leaves had higher free amino acid contents than control leaves. This result agrees well with previous findings of several researchers (Bestman *et al.* 1990a, Brunk and Rhodes 1988, Rhodes *et al.* 1987, Royuela *et al.* 1991), who concluded that chlorsulfuron-treated plants had increased free amino acids, probably due to a reduction in utilization of amino acids to protein

(Bestman *et al.* 1990a) and/or an increase in proteolysis (Brunk and Rhodes 1988, Rhodes *et al.* 1987, Royuela *et al.* 1991). When the rate of protein hydrolysis is higher than the rate of protein synthesis because of an increase in proteolysis, it is expected that chlorsulfuron-treated leaves have higher amino acid content and lower protein content than control leaves. However, protein concentrations in treated canola leaves between 12 and 48 h after chlorsulfuron treatment were similar to those in control leaves. Furthermore, protein profiles in chlorsulfuron-treated canola leaves were not different from those in control leaves. However, it might be a premature conclusion that an increase in free amino acids in chlorsulfuron-treated canola leaves is not due to an increase in proteolysis since protease activity or individual amino acid contents were not measured in this research.

Comparisons of data on changes in free amino acid and carbohydrate contents of the treated canola leaves showed that the free amino acid content changed prior to changes in carbohydrates, suggesting that carbon might be allocated favorably to amino acids. Carbon allocation between sucrose and amino acids is regulated, in part, by activation of PEPcase and inactivation of SPS (Champigny and Foyer 1992, Quy 1991). This mechanism is controlled by nitrate, which activates cytosolic protein kinases. In chlorsulfuron-treated canola leaves, however, there was no activation of PEPcase (Fig. 23) and no inactivation of SPS (Fig. 22) 24 h after herbicide treatment. Therefore, although the nitrate concentration in the cytoplasm was not measured in chlorsulfuron-treated canola leaf tissues, it is reasonable to conclude that the accumulation of amino acids in the treated leaves is not due to nitrate-induced carbon allocation. Carbon allocation between sucrose and amino acids is also controlled, in part, by NR. The activation of NR increases carbon allocation to amino acids by provision of carbon backbones and energy (Rufty *et al.* 1992) and, consequently, leads to a reduction in sucrose synthesis (Foyer *et al.* 1994, Lang *et al.* 1993, Stulen 1986). In chlorsulfuron-treated canola leaves, NR activity was higher than in control leaves (Fig. 24). However, increases in NR activity occurred much later (21 h after treatment) than the reduction in sucrose export (12 h after treatment), indicating that the reduction of sucrose transport out of chlorsulfuron-treated canola leaves is not due to changes in carbon allocation, which were expected to increase amino acid content and reduce sucrose content.

How did chlorsulfuron-treated canola leaves maintain a higher level of NR activity than control leaves? Cheng *et al.* (1992) reported that etiolated *Arabidopsis* increased levels of NR mRNA in the presence of sucrose, indicating an induction of NR by sucrose. One might speculate that, in chlorsulfuron-treated canola leaves, sucrose that was not transported induces NR mRNA and subsequently increases NR activity. If this occurs in chlorsulfuron-treated canola leaves, an interesting scenario can develop. First, chlorsulfuron reduces sucrose export out of treated leaves (12 h after treatment). The sucrose that is not exported induces NR mRNA which, in turn, increases NR. As a consequence, NR activity increases (18 h after treatment). With an adequate supply of substrate (nitrate), the NR produces more amino acids (24 h after treatment). In addition to sucrose, light, nitrate and plant growth regulators are known to increase NR activity (Beevers and Hageman 1972, Hoff *et al.* 1992, Li and Oaks 1993, Mohr *et al.* 1992). The effects of chlorsulfuron were not light-dependent (Fig. 24, A); therefore, the increase in NR of chlorsulfuron-treated canola leaves probably is not associated with the light-induced NR activation. Nitrate (Champigny and Foyer 1992, Quy 1991) activates PEPcase and inactivates SPS by activation of protein kinases, resulting in an increase in amino acid synthesis and a decrease in sucrose synthesis. However, there was no activation of PEPcase (Fig. 23) and no inactivation of SPS (Fig. 22) in chlorsulfuron-treated canola leaves. Therefore, it is difficult to accept a concept that nitrate is involved in NR activation in chlorsulfuron-treated canola leaves.

Overall results from carbon fixation and carbohydrate production and allocation in chlorsulfuron-treated canola leaves indicate that a reduction in sucrose transport is not due to an inability of the leaves to produce carbohydrate, or to a change in carbon allocation in the leaves. In chlorsulfuron-treated canola leaves, therefore, changes in carbon metabolism were subsequent results following a reduction in sucrose transport. How did chlorsulfuron inhibit sucrose transport in treated leaves? Since the herbicidal effects of chlorsulfuron in treated canola seedlings were prevented partially by supplementation with BCAA and PA (Table 2 in section 4.1.3), the inhibition of sucrose transport in chlorsulfuron-treated canola leaves is believed to be related to a shortage of BCAA and PA. Dysfunction of proteins that are involved in sucrose transport into the phloem (Bestman *et al.* 1990a, Lowther 1990) has been suggested as an inhibitory mechanism. However, as discussed earlier in section 4.2.4, this mechanism has shortcomings, and more work is needed to elucidate the connection between a site of action of chlorsulfuron and a reduction of sucrose transport.

In conclusion, twelve hours after chlorsulfuron treatment, when the treated leaves exported less sucrose in chlorsulfuron-treated canola leaves, there was no difference between control and chlorsulfuron-treated leaves in the metabolic and physiological processes examined. By 24 h after treatment, the treated leaves had higher NR activity and higher free amino acid contents. Although there is a chance of increase in free amino acid content by an increase in NR activity, it is not known whether the increased free amino acid synthesis changed carbon allocation to reduce sucrose synthesis and, consequently, reduced sucrose transport.

Nothwithstanding the changes in NR activity and free amino acid content 24 h after treatment, chlorsulfuron did not affect carbon fixation and its related processes, and carbohydrate production in chlorsulfuron-treated canola leaves. The treated leaves had similar levels of chlorophyll, NCE and transpiration rates, and sugars such as glucose, fructose, sucrose, total sugar, and starch. In addition, SPS activity of chlorsulfuron-treated canola leaves was similar to that of control leaves. Results from carbon dioxide fixation and metabolism, and carbohydrate production, indicate that chlorsulfuron-treated leaves produced carbohydrates at a level comparable to that of control leaves 24 h after treatment. In addition, enzyme activities of chlorsulfuron-treated canola leaves which are involved in carbon allocation between sucrose and amino acids were not different from those in control leaves. Therefore, a reduction in sucrose transport in chlorsulfuron-treated canola leaves is not due to an inability of treated leaves to produce carbohydrates.

Twenty-four hours after treatment, respiration rates of chlorsulfuron-treated leaves were not different from those of control leaves. However, during an eight-hour experiment, chlorsulfuron-treated leaves had a higher respiration rate, which might indicate that the treated leaves had a higher carbohydrate pool size than control leaves. The carbohydrate pool size of chlorsulfuron-treated canola leaves was changed 48 h after treatment. Chlorsulfuron-treated canola leaves that exported less sucrose than control leaves had higher levels of glucose, total sugar, and starch contents than control leaves. Since chlorsulfuron reduced sucrose transport out of treated leaves (source), the actively growing plant parts (sinks) are short of carbohydrate. The carbohydrate shortage in sink tissues of chlorsulfuron-treated canola seedlings can, in part, reasonably explain the growth reduction of treated plants.

4.4. Effect of chlorsulfuron on sucrose transport into a sink

Chlorsulfuron-treated canola seedlings showed a reduction in sucrose translocation (4.2). In this section, the aim was to examine if reduced sucrose translocation in chlorsulfuron-treated canola seedlings was related to reduced sink strength.

4.4.1. Sink-to-source transition of the third leaf of canola seedlings

The area and weight of the third leaf of canola seedlings were measured to determine when the third leaf changed from a sink to a source.

The third leaf was first visible 8 days after transplanting (DATP). The growth pattern of the third leaf was similar to that of the first leaf, but the growth rates were different (Fig. 25) (first leaf data are in Fig. 11). Leaf weight data showed the same pattern as leaf area (data not shown). Leaf area increased linearly at the rate of 8.5 cm^2 d^{-1} between 11 and 16 DATP, at which time the maximum leaf area was attained. ^{14}C transport, following ^{14}C-sucrose application, out of the third leaf increased after 14 DATP (Fig. 25). The results from this experiment suggest that a sink-to-source transition occurred between 13 and 14 DATP, on the basis of a suggestion by Dale (1992), who concluded that a sink-to-source transition occurs when a leaf reaches about 30-35% of the final leaf area or the final lamina length. The results from this experiment imply that the third leaf of canola seedlings predominantly imports assimilates before 14 DATP and exports assimilates after 14 DATP. In subsequent experiments, therefore, chlorsulfuron and ^{14}C-sucrose were applied 13 or 14 DATP when the third leaf began to change to a source.

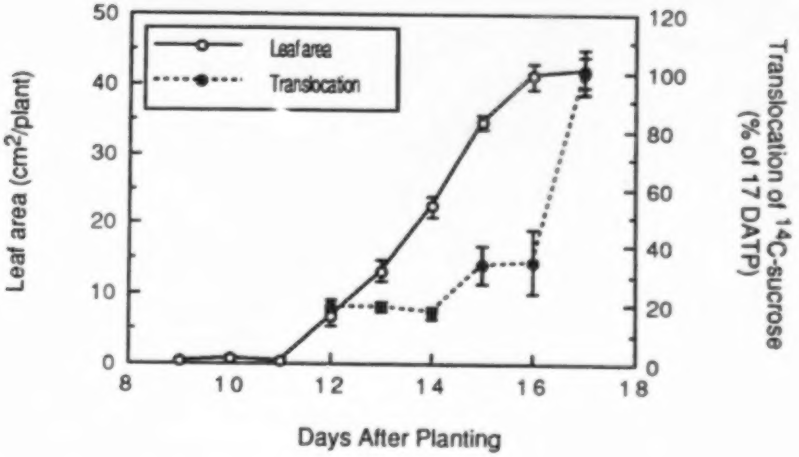

Figure 25. Sink-to-source transition of the third leaf of canola seedlings. The leaves were harvested between 9 and 17 DATP. Means and standard errors of leaf area are based on data from ten replicates. Radio-labeled sucrose was applied to the leaves and ^{14}C export was determined after 24 h. Means and standard errors of ^{14}C translocation are based on data from four replicates.

4.4.2. Effect of chlorsulfuron on sink strength

4.4.2.1. Sink size

Canola seedlings at the four-leaf stage were modified by leaf excision and stem girdling to have one source (the first leaf) and one sink (the third leaf). Seventy-two hours after leaf excision and stem girdling, the seedlings had 25% higher shoot dry weight than unmodified seedlings (400 mg vs 320 mg) but 56% lower root dry weight (60 mg vs 140 mg), suggesting that the modification process promoted shoot growth. The sink leaves were treated with various amounts of chlorsulfuron 48 h after modification. The sink size of the leaves was determined 24 h after treatment by measuring dry weights and protoplast numbers.

Dry weight of control sink leaves was 124 mg after 24 h (Fig. 26). The dry weight was reduced by approximately 40% by chlorsulfuron treatment at dosages of 0.25 µg or more. On the basis of these results, the sink leaves were treated with 0.25 µg of chlorsulfuron in further experiments of this section. The absorption rate of chlorsulfuron by the sink leaves was not examined. It is not known, therefore, how much of the applied chlorsulfuron was in tissues of the sink leaves. Assuming the third leaf absorbs chlorsulfuron at the same rate as the first leaf [31.5% at 24 h, Appendix 3], the third leaf contained 0.08 µg of chlorsulfuron 24 h after application. When 1 µg of chlorsulfuron was applied to the first leaf of canola seedlings, 0.004 µg of chlorsulfuron was found in the shoot apex [0.4% of total applied amount] (Appendix 3). This indicates that the amount of chlorsulfuron in the third leaf in this experiment was approximately 20 times higher than that in other experiments in which the first leaf of seedlings was treated with chlorsulfuron.

Protoplasts extracted from control leaves are illustrated in Fig. 27. Application of 0.25 µg chlorsulfuron reduced the number of protoplasts by 82% compared to control leaves (Fig 28). Considering the absence of a dose-response of dry weight to chlorsulfuron (Fig. 26), a dose response of the number of protoplasts to chlorsulfuron was not performed.

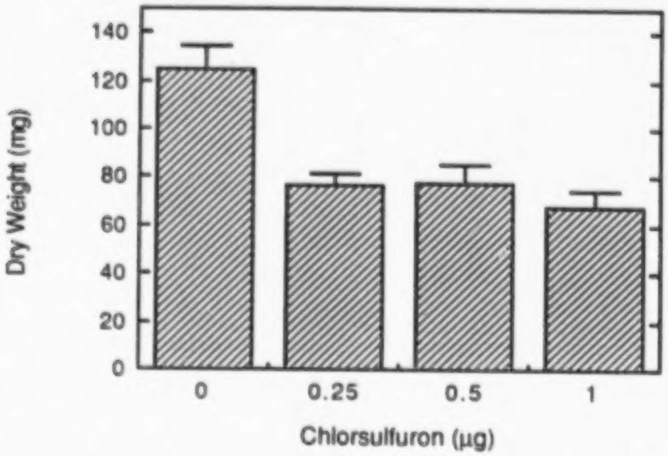

Figure 26. Effect of chlorsulfuron on dry weight of the third leaf of modified canola seedlings. Forty-eight hours after modification, chlorsulfuron was applied to the third leaf. Twenty-four hours later, dry weight was taken as a measure of sink size. Means and standard errors are based on data from six replicates.

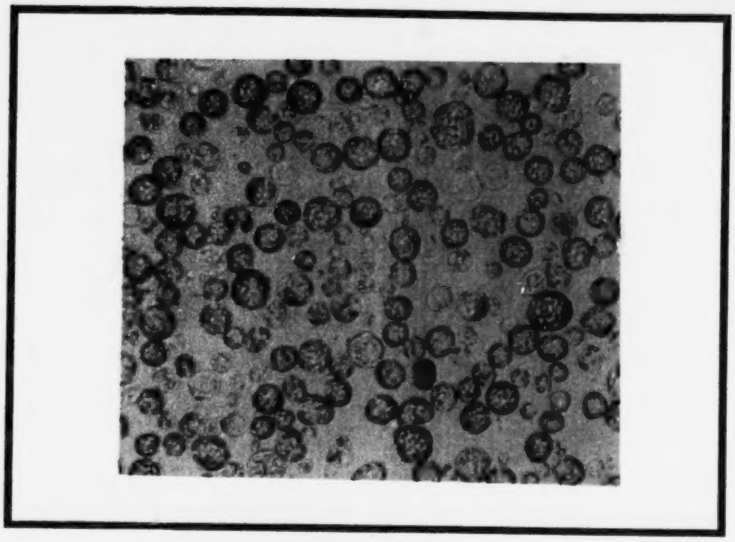

Figure 27. Protoplasts extracted from the third leaf of modified canola seedlings at the four-leaf stage.

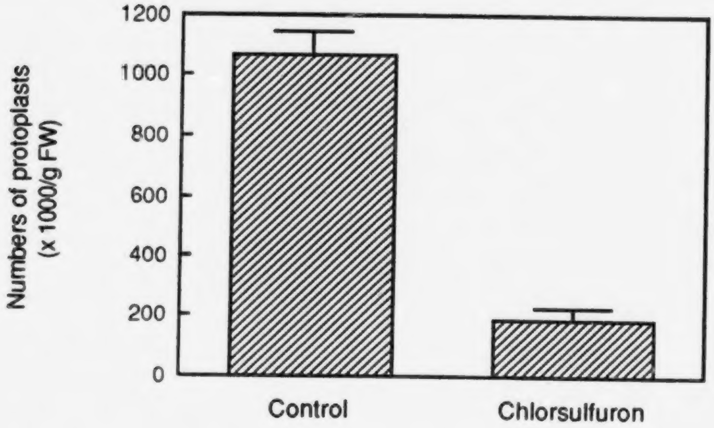

Figure 28. Effect of chlorsulfuron on the number of protoplasts. Protoplasts were extracted from the third leaf of modified canola seedlings. The number of protoplasts was taken as a measure of sink size. Means and standard errors are based on data from two replicates.

4.4.2.2. Sink activity

The sink leaves of modified seedlings were treated with 0.25 µg of chlorsulfuron. Sink activity was determined by measuring ^{14}C uptake into leaf discs taken from the sink leaves.

Uptake of ^{14}C in 30 min by leaf discs from chlorsulfuron-treated leaves was not different from that by leaf discs from control leaves (Table 8). However, the uptake of ^{14}C by a cell of chlorsulfuron-treated leaves was 4-fold higher than that by a cell of control leaves (Table 8), suggesting that chlorsulfuron promoted uptake of assimilates. The sucrose uptake of leaf discs, however, did not reflect the sink activity for sucrose uptake because of leaf size differences between control and chlorsulfuron-treated leaves. Dry weight of control leaves was almost 2 times higher than that of chlorsulfuron-treated leaves (124 mg vs 67 mg). Considering the similar ^{14}C uptake ability of leaf discs from both control and chlorsulfuron-treated leaves, the treated leaves could import less sucrose.

4.4.3. Effect of chlorsulfuron on selected metabolites

The sink leaves of the modified seedlings were treated with 0.25 µg chlorsulfuron. The contents of total sugar and free amino acids in the source and sink leaves of control and chlorsulfuron-treated seedlings were determined 24 h after treatment, at which time the sink strength was measured.

Total sugar content in the source and sink leaves of control seedlings was 54 and 92 nmoles glucose equivalent mg^{-1} FW, respectively (Fig. 29, A). Application of chlorsulfuron to the sink leaves had no effect on the total sugar content in either the source or sink leaves.

Free amino acid content in the source and sink leaves of control seedlings was 6 and 9 nmoles leucine equivalent mg^{-1} FW, respectively (Fig. 29, B). The free amino acid content in the source and sink leaves of chlorsulfuron-treated seedlings was 33% and 300% higher, respectively, than that in control seedlings.

Table 8. Sink activity of the third leaves of modified canola seedlings with and without chlorsulfuron treatment. The sink activity was measured by uptake of sucrose into leaf discs for 30 min. Results are based on three replicates.

Treatment	Uptake of ^{14}C	Uptake of ^{14}C per cell[1]
	(dpm/g FW/h)	(dpm/cell/h)
Control	44450	0.04
Chlorsulfuron	31594 [ns]	0.17

[1] calculated on the basis of data from Fig. 28.

ns not significant at $P < 0.05$

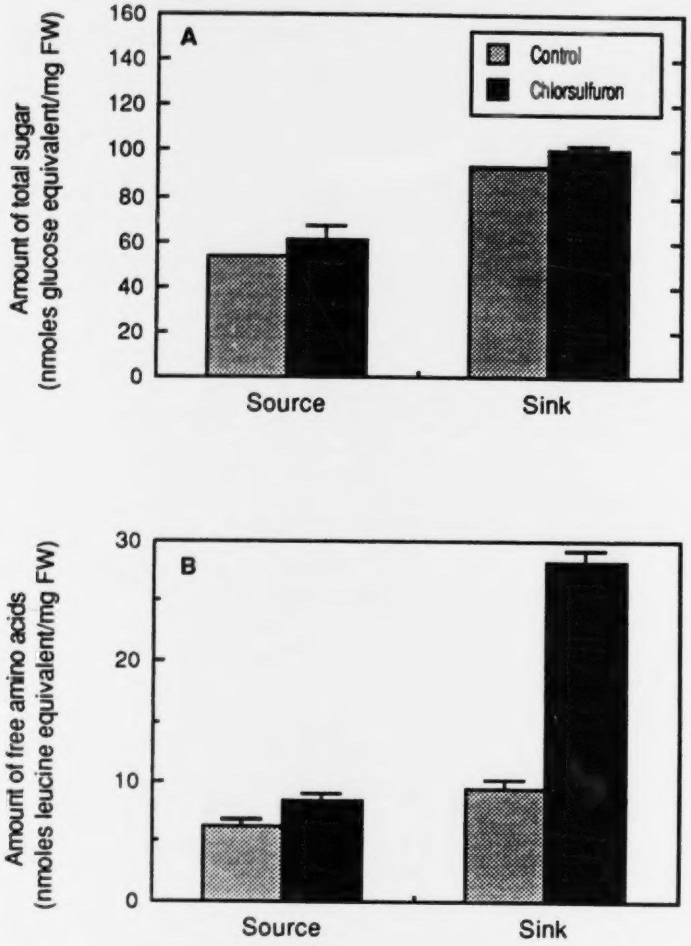

Figure 29. Effect of chlorsulfuron on selected metabolites in the source and sink leaves of modified canola seedlings. A, total sugar; B, free amino acids. Means and standard errors are based on data from six replicates.

4.4.4. Effect of reduced sink strength on sucrose translocation

4.4.4.1. Effect of sink treatment with chlorsulfuron on exudation of sucrose from source leaves of the modified canola seedlings

Sink leaves of modified canola seedlings were treated with 0.25 µg of chlorsulfuron. Twenty-four hours after treatment, the source leaves were treated with ^{14}C-sucrose. The source leaves then were excised and allowed to exude sugars including ^{14}C. Excised leaves of chlorsulfuron-treated seedlings exuded 78% more ^{14}C than leaves of control seedlings (Table 9).

4.4.4.2. Translocation of ^{14}C in modified canola seedlings

Sink leaves of modified seedlings were treated with 0.25 µg chlorsulfuron. Twenty-four hours after treatment, source leaves were treated with ^{14}C-sucrose. Translocation of ^{14}C was measured to determine if chlorsulfuron applied to sink leaves affected assimilate translocation.

Application of chlorsulfuron to the sink leaves reduced ^{14}C transport out of the source leaves from 28% of the recovered ^{14}C to 12 to 16%, depending on the dose of chlorsulfuron (Fig. 30).

4.4.5. Discussion

Sink strength is comprised of sink size and sink activity (Warren-Wilson 1967) and refers to the rate of accumulation or utilization of assimilates in actively growing parts of plants (Patrick 1993, Doehlert 1993). Data from sucrose uptake showed that the leaf discs from sink leaves of control and chlorsulfuron-treated canola seedlings took up similar amounts of sucrose (Table 8), suggesting that chlorsulfuron did not reduce sink activity of the treated leaves. It should be noted that the leaf discs contained cut ends which could allow sucrose access to cells without any

Table 9. Exudation of ^{14}C out of excised source leaves of modified canola seedlings. Results are based on eight replicates.

Treatment	Exudation of ^{14}C	Effect of Herbicide
	(dpm/g FW/min)	(% of control)
Control	277	
Chlorsulfuron	494 *	178

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* The effect of chlorsulfuron is significant at $P < 0.01$.

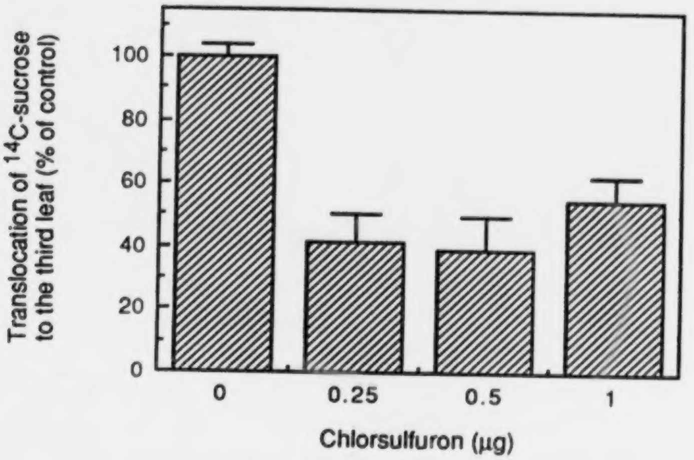

Figure 30. Effect of chlorsulfuron on ^{14}C translocation into the third leaf of modified canola seedlings. The third leaf was treated with chlorsulfuron and the first leaf was treated with ^{14}C-sucrose 24 h later. A further 24 h later, ^{14}C activity in the third leaf was measured. Means and standard errors are based on data from six replicates.

metabolic uptake process. The results of sucrose uptake by leaf discs, therefore, could be an under-estimation of the effect of chlorsulfuron on sucrose import. Because of this problem, the sink activity was calculated on the basis of cell numbers. Cells of chlorsulfuron-treated sink leaves contained more ^{14}C than those of control sink leaves, considering the number of cells in the sink leaves. The uptake of ^{14}C by a cell of chlorsulfuron-treated leaves was 4-fold higher than that by a cell of control leaves, suggesting that chlorsulfuron did not inhibit the enzyme complement or metabolism involved in uptake and utilization of assimilates. However, it is also arguable whether uptake of assimilates by cells represents the ability of the whole sink tissue that may contain a dozen or more types of cells (Farrar 1993), each of which has different enzyme complements and metabolism. It might be more reasonable, therefore, to consider the assimilate-importing ability of sink leaves as Warren-Wilson (1967) has suggested. The average dry weights of control and chlorsulfuron-treated sink leaves were 124 mg and 67 mg, respectively. Assuming that dry weight represents fresh weight of the sink leaves, the calculated ^{14}C uptake by control and chlorsulfuron-treated leaves was 5512 and 2117 dpm mg^{-1} DW h^{-1}, respectively. These results indicate that chlorsulfuron applied directly to sink leaves reduced their sink activity.

In addition, both leaf weight (Fig. 26) and number of protoplasts (Fig. 28) in the sink leaves were reduced by chlorsulfuron, supporting the conclusion that chlorsulfuron reduced sink size. Although chlorsulfuron reduced both sink size determinants, the reduction was greater in protoplast numbers (82%) than in dry weight (40%). The protoplast number reflects cell division while dry weight reflects cell growth which is composed of both cell division and cell elongation. Therefore, the greater reduction of protoplast numbers in chlorsulfuron-treated sink leaves of canola seedlings indicates that the herbicide effect is mainly through cell division inhibition rather than cell elongation inhibition. This agrees well with the previous findings of Ray (1980), who reported that chlorsulfuron did not affect cell elongation in pea, cucumber and lettuce. Inhibition of cell division in chlorsulfuron-treated plants has been reported by other researchers (Ray 1984, Rost 1984, Rost and Reynolds 1985, Robbins and Rost 1987). The mechanisms connecting the site of action of chlorsulfuron with inhibition of cell division, however, are not known. In conjunction with the site and mechanism of action of chlorsulfuron, three possible mechanisms could be related to inhibition of cell division: (1) a shortage of certain proteins, such as cycline-dependent kinase and cyclines (Doerner 1994) that are involved in cell cycle transition from G1 to S and from G2 to M, (2) a disturbance in levels of polyamines (DiTomaso 1988, Giardina and Carosi 1990) that are involved in the regulation of cell cycle progression (Evans and Malmberg 1989), and (3) an accumulation of toxic metabolites such as 2-ketobutyrate and 2-aminobutyrate that inhibited cell division in microorganisms (LaRossa *et al.* 1987) and plants (Lanzagorta *et al.* 1988). Changes in metabolites in the first leaves of 2-ketobutyrate-treated canola seedlings were different from those in chlorsulfuron-treated plants (Fig. 9 and 10 in 4.1.4); therefore, the involvement of 2-ketobutyrate and 2-aminobutyrate is questionable.

Application of chlorsulfuron to the sink leaves of modified canola seedlings reduced both sink size and sink activity, indicating that chlorsulfuron reduced sink strength. Many researchers have shown that the reduction of sink strength by physical modifications or chemical inhibitors resulted in reduced assimilate translocation (Ho 1988, Marowitch *et al.* 1986, Paul *et al.* 1990, van Oene *et al.* 1992a, van Oene *et al.* 1992b). The mechanisms involved in the reduction of assimilate translocation by reduced sink strength are not clearly understood, although an osmotic effect in the sinks controls the unloading of assimilates (Grusack and Minchin 1988). The canola seedlings with lower sink strength transported less ^{14}C-sucrose from treated source leaves to sink leaves (Fig. 30). This could result in the accumulation of transporting carbohydrates in the source leaves, and could account for greater exudation of ^{14}C-sucrose by excised source leaves of chlorsulfuron-treated seedlings (Table 8). Since chlorsulfuron was applied directly to the sink leaves, a herbicide effect could be limited to the sink leaves. In the adjacent-leaf experiment, a similar experimental situation to the modified-plant experiment was simulated (section 4.2.2). In the modified plants where 0.25 µg of chlorsulfuron was directly applied to the sink leaves, 12% of the total recovered radioactivity had been exported (Fig. 30), while in the intact plants in which 0.004 µg of chlorsulfuron was detected in the sink leaves (refer to 4.4.2.1), 34% of total recovered radioactivity had been exported (Table 4). The differences in sucrose export between the experiments could be due to the amount of chlorsulfuron in the sink leaves, suggesting that the more chlorsulfuron is translocated to sinks, the less sucrose is exported from source leaves.

The free amino acid content in chlorsulfuron-treated sink leaves increased by 300% compared to that in control sink leaves (Fig. 29, B). Chlorsulfuron-treated sink leaves of modified seedlings showed a different pattern of protein bands from control leaves. In SDS-PAGE analysis of chlorsulfuron-treated sink leaves, new protein bands appeared in a high molecular weight region and some major protein bands disappeared in a low molecular weight region (Appendix 6), suggesting protein turnover. The increase in amino acid content in chlorsulfuron-treated sink leaves, therefore, is probably due to protein hydrolysis as suggested by Brunk and Rhodes (1988).

In conclusion, chlorsulfuron-treated sink leaves of modified canola seedlings had lower sink size and activity, determinants of sink strength. The sink strength reduction by chlorsulfuron reduced ^{14}C transport out of the source leaf of the modified canola seedlings. These results suggest that reduced sucrose translocation in chlorsulfuron-treated canola seedlings is related to reduced sink strength.

4.5. Effect of high CO_2 concentrations on sucrose transport out of excised canola leaves

Chlorsulfuron-treated excised canola leaves exported less sucrose than untreated leaves (section 4.2). The aim of this series of experiments was to examine whether exposure of the leaves to high CO_2 concentrations could overcome the reduction in sucrose transport.

4.5.1. Maximum net carbon exchange

The first leaves of seedlings at the four-leaf stage were excised and cut petiole ends were placed in phosphate buffer solution containing EDTA. The excised leaves were exposed to ambient (350 $\mu l\ l^{-1}$) and above-ambient (500 and 900 $\mu l\ l^{-1}$) CO_2 to determine their response to high CO_2.

Maximum net carbon exchange (NCE) rates at above-ambient CO_2 levels were 77% to 91% higher than at ambient CO_2 (Fig. 31). These maximum rates were higher than the initial rates (immediately after leaf excision) and were reached within 75 min (data not shown; patterns over time were similar to those in Fig. 16, B). NCE rates at 500 and 900 $\mu l\ l^{-1}$ CO_2 did not differ.

4.5.2. Net carbon exchange and transpiration rates

The first leaves of seedlings at the four-leaf stage were treated with chlorsulfuron. Control and chlorsulfuron-treated leaves were excised and were exposed to ambient (350 $\mu l\ l^{-1}$) and high (500 $\mu l\ l^{-1}$) CO_2. The NCE and transpiration rates of the leaves were measured.

The NCE rate of control leaves was higher at high CO_2 than at ambient CO_2 (Fig. 32). Exposure of control leaves to high CO_2 increased the maximum NCE rate by 47% over that at ambient CO_2 200 min after leaf excision. The high CO_2 effect was maintained during the length of the experiment (8 h). The NCE rate of chlorsulfuron-treated leaves at high CO_2 was higher than at

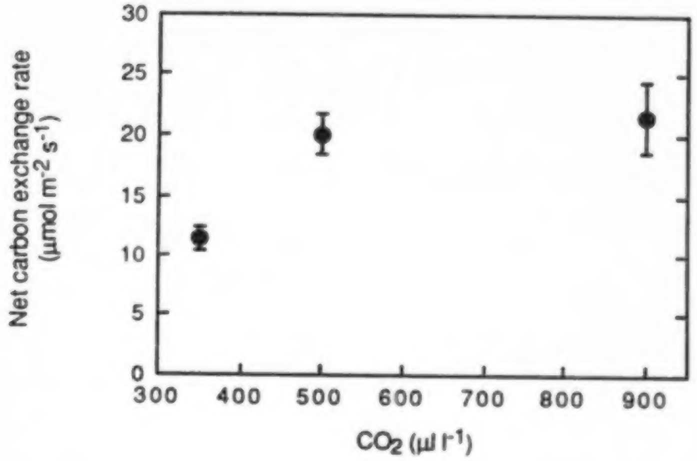

Figure 31. Maximum net carbon exchange rate of excised canola leaves at ambient and above-ambient CO_2 concentrations. The leaves were exposed to 350, 500, and 900 µl l^{-1} CO_2. Means and standard errors are based on data from three to five replicates.

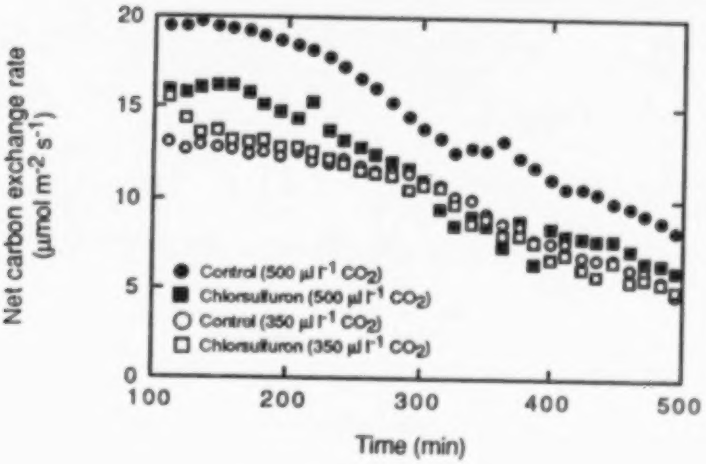

Figure 32. Net carbon exchange rates of excised canola leaves at above-ambient levels of CO_2. Data were collected every 12 min for 8 h. The data plotted are based on the results of three replicates. At 500 μl l^{-1} CO_2, *F*-values for the main effects of chlorsulfuron and time were significant at the $P_{0.01}$ level.

ambient CO_2 (14.4 vs 12.8 µmol m^{-2} s^{-1} 200 min after leaf excision) (Fig. 32). However, the high CO_2 effect was maintained only until 250 min after leaf excision.

Control leaves at high CO_2 showed higher transpiration rates than at ambient CO_2 (Fig. 33). The rate was 72% higher than that at ambient CO_2 200 min after leaf excision. However, the transpiration rate of chlorsulfuron-treated leaves at high CO_2 was similar to that at ambient CO_2.

4.5.3. Selected metabolites

The contents of starch, total sugars, and free amino acids in control and chlorsulfuron-treated canola leaves were determined after measuring NCE and transpiration rates (section 4.5.2).

After 8 hours' exposure to ambient CO_2, contents of both starch (Fig. 34, A) and total sugar (Fig. 34, B) in control and chlorsulfuron-treated leaves were similar, whereas the content of free amino acids (Fig. 34, C) in chlorsulfuron-treated leaves was four times higher than in control leaves.

After 8 hours' exposure to high CO_2, the starch content of control leaves was 58% higher than in leaves at ambient CO_2 (Fig. 34, A). The high-CO_2-induced increase in starch content in chlorsulfuron-treated leaves was lower (24% vs 58%). Control leaves exposed to high CO_2 had levels of total sugar and amino acids comparable to those at ambient CO_2. The inhibition of photorespiratory carbon metabolism at high CO_2 could possibly account for an absence of the high CO_2 effect on levels of free amino acids in the leaves. The amounts of total sugars and amino acids of chlorsulfuron-treated leaves at high CO_2 had not been affected significantly due to high variation. However, in both CO_2 conditions, the amino acid content of chlorsulfuron-treated leaves was higher than that of control leaves, presumably due to an increase in proteolysis and/or an impaired utilization of amino acids in protein synthesis.

The results indicate that additionally synthesized carbohydrate at high CO_2 partitioned favorably to starch in both control and chlorsulfuron-treated leaves.

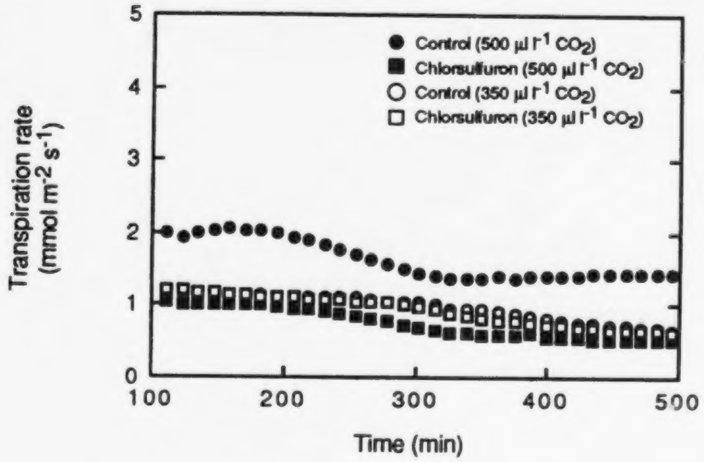

Figure 33. Transpiration of excised canola leaves at above-ambient level of CO_2. The leaves were exposed to 500 µl l^{-1} CO_2. Data were collected every 12 min for 8 h. The data plotted are based on the results of three replicates. *F*-values for the main effects of chlorsulfuron and CO_2 and their interaction were all significant at the $P_{0.01}$ level.

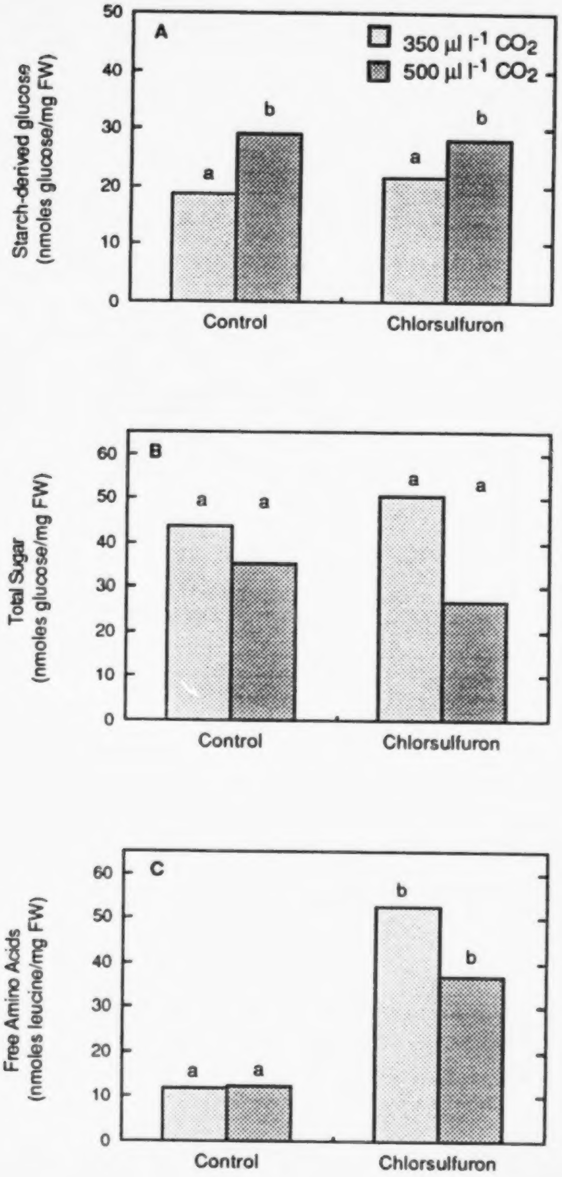

Figure 34. Changes in selected metabolites of control and chlorsulfuron-treated excised canola leaves exposed to 350 and 500 µl l^{-1} CO_2. A, starch content. *F*-value for CO_2 concentration was significant at the $P_{0.05}$ level; B, total sugar content. *F*-values for chlorsulfuron and CO_2 concentration were not significant at the $P_{0.05}$ levels; C, free amino acid content. *F*-value for chlorsulfuron was significant at the $P_{0.01}$ level.

4.5.4. Sucrose exudation

Sucrose content of exudates from control and chlorsulfuron-treated excised leaves, exposed to ambient and high CO_2, was determined enzymatically. Control leaves at ambient CO_2 exuded sucrose at the rate of 12.2 nmoles g^{-1} FW h^{-1} (Fig. 35). At high CO_2, control leaves exuded similar amount of sucrose to those at ambient CO_2.

Chlorsulfuron-treated leaves exuded less sucrose than control leaves at both ambient and high CO_2 levels. At ambient CO_2, chlorsulfuron-treated leaves exuded sucrose at the rate of 5.7 nmoles g^{-1} FW h^{-1} (62% of control) (Fig. 35). Chlorsulfuron-treated leaves at high CO_2 exuded sucrose at about the same rate as those at ambient CO_2.

4.5.5. Discussion

Short-term exposure of many C_3 plants to high CO_2 concentrations increases the net carbon exchange rate (Arp 1991, Eamus and Jarvis 1989, Farrar and Williams 1991, Grodzinski 1992, Mooney *et al.* 1991) and reduces the transpiration rate and leaf conductance (Mooney *et al.* 1991). Leaves of plants exposed to high CO_2 for a short period had higher levels of starch (Yelle *et al.* 1989, Cave *et al.* 1981, Wulff *et al.* 1982) and sugars (Geiger *et al.* 1983, Hoddinott and Jolliffe 1988), and the leaves transported more assimilates (Geiger *et al.* 1983). Similarly, in the research described here, excised canola leaves exposed to 500 µl l^{-1} CO_2 for 8 h responded with an increase in NCE rate. Leaves exposed to high CO_2 had higher levels of starch but had a similar level of total sugar. The leaves, however, did not increase sucrose exudation compared to the leaves at ambient CO_2, indicating that exposure of excised canola leaves to high CO_2 increased only carbohydrate production, not translocation.

In contrast to other plants, the exposure of excised canola leaves to high CO_2 increased the transpiration rate. High CO_2 is known to reduce stomatal conductance by a process of negative feedback (Raske 1975); an increase of atmospheric partial pressure of CO_2 caused an

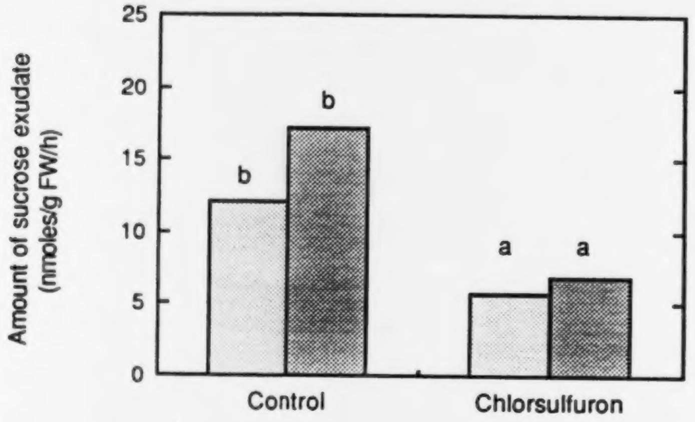

Figure 35. Sucrose exudation out of control and chlorsulfuron-treated excised canola leaves exposed to 350 and 500 µl l^{-1} CO_2. *F*-value for the main effect of chlorsulfuron was significant at the $P_{0.05}$ level.

increase in intercellular partial pressure of CO_2 which, in turn, reduced the stomatal conductance. This implies that leaves start to close stomata when the intercellular partial pressure of CO_2 is higher than a certain threshold level. If it is true, the intercellular partial pressure of CO_2 in the excised canola leaves at high CO_2 might not reach a certain threshold level for stomata closure. This, however, does not explain why the transpiration rate at high CO_2 was higher than at ambient CO_2.

Chlorsulfuron-treated excised canola leaves exposed to 500 $\mu l\ l^{-1}$ CO_2 also had a higher NCE rate than at ambient CO_2. However, this effect on the NCE rate lasted only a few hours. The absence of a high CO_2 effect after that is believed to be linked to herbicide action, but the nature of the mechanism linking site or mechanism of action of chlorsulfuron with absence of a high CO_2 effect has not emerged in this research. Although at high CO_2 the leaves had a slightly lower transpiration rate than at ambient CO_2, the impact of such a reduction in transpiration rate on the NCE rate might be minimal. In maize, water-stress-induced stomatal closure has been observed to begin when leaf water potential falls to a certain threshold level (Beadle *et al.* 1973). The cut petiole ends of both control and chlorsulfuron-treated excised canola leaves were placed in buffer solution. However, it is not known whether the treated leaves decreased their water potential to a certain threshold which can close stomata and, as a consequence, reduce transpiration.

Presumably as a consequence of the increase in NCE rate, chlorsulfuron-treated leaves at high CO_2 accumulated starch. How did chlorsulfuron-treated excised canola leaves change carbohydrate partitioning to produce more starch? It is known that an increase in 3-phosphoglycerate in chloroplasts and a reduction in inorganic phosphate (Pi) in cytoplasm can increase the rate of ADPglucose synthesis and, hence, starch synthesis (Hawker *et al.* 1991, Stitt and Quick 1989). Since cut petiole ends of excised leaves were placed in phosphate buffer solution, the leaves are expected at least not to be short of inorganic phosphate (refer to Materials and Methods), suggesting that the favorable starch synthesis in chlorsulfuron-treated leaves at high CO_2 might be due to a buildup of 3-phosphoglycerate.

If chlorsulfuron reduces the ability of treated leaves to load assimilates into the phloem

(Bestman *et al.* 1990a, Hall and Devine 1993, Vanden Born *et al.* 1988), the fate of sucrose is expected to be changed in chlorsulfuron-treated leaves at both ambient and high CO_2 levels. Data from the sugar analysis (Table 5 in section 4.3.5) showed that the treated leaves at ambient CO_2 did not accumulate sucrose but hexoses including glucose, suggesting that sucrose that was not exported was stored as hexoses in canola seedlings. The treated leaves at high CO_2, however, had a similar level of hexoses to those at ambient CO_2, suggesting that additionally fixed carbon at high CO_2 partitioned favorably to starch. Therefore, the reduction of sucrose transport in chlorsulfuron-treated canola leaves exposed to high CO_2 might be, in part, due to altered carbon allocation.

Madore and Grodzinski (1985) reported that at high CO_2, photorespiratory carbon metabolism decreased pools of glycine and serine, which resulted in reduction of carbohydrate partitioning to amino acids. Excised canola leaves exposed to high CO_2 had levels of free amino acids comparable to those at ambient CO_2. These results suggest that such leaves reduced photorespiratory carbon metabolism at high CO_2. In contrast, chlorsulfuron-treated leaves had higher levels of free amino acids, probably due to proteolysis (Brunk and Rhodes 1988, Rhodes *et al.* 1987, Royuela *et al.* 1991) and/or to impaired utilization of amino acids to protein (Bestman *et al.* 1990a). In chlorsulfuron-treated canola leaves, nitrate reductase activity increased (Fig. 24, A in section 4.3.9.3).

In conclusion, exposure of chlorsulfuron-treated excised canola leaves to 500 $\mu l\ l^{-1}$ CO_2 did not overcome the reduction in sucrose transport. Although the leaves had a slightly lower transpiration rate, chlorsulfuron-treated leaves exposed to high CO_2 increased in NCE rate and carbohydrate production. Apart from a reduction in photorespiratory carbohydrate metabolism, chlorsulfuron-treated leaves accumulated free amino acids, presumably by proteolysis and/or impaired utilization of amino acids. Chlorsulfuron-treated leaves at high CO_2 partitioned the newly fixed carbon mainly to starch, a storage carbohydrate, suggesting that the leaves partitioned relatively less carbon to sucrose, a transporting carbohydrate. Therefore, the reduction in sucrose transport out of chlorsulfuron-treated leaves at high CO_2 may be, in part, due to a reduction of precursors for sucrose synthesis.

5. SUMMARY AND CONCLUSIONS

Canola seedlings were susceptible to chlorsulfuron, and a dose of 1 µg per plant reduced their growth by about 50%. In addition, treated seedlings showed injury symptoms such as chlorosis, leaf rolling, and accumulation of anthocyanins. Supplementation with branched-chain amino acids or branched-chain amino acids plus pantothenic acid could, at least partially, prevent chlorsulfuron-induced growth reduction. Supplementation with branched-chain amino acids prevented at least part of the chlorsulfuron-induced growth reduction but did not prevent chlorosis in the young leaves. Supplementation with both branched-chain amino acids and pantothenic acid resulted in partital prevention of both growth inhibition and chlorosis. The results suggest that the chlorsulfuron-induced phytotoxicity is associated with a shortage of both the branched-chain amino acids and pantothenic acid. Canola seedlings treated with 2-ketobutyrate changed metabolites in a different manner than plants treated with chlorsulfuron. The first leaves of 2-ketobutyrate-treated seedlings had similar concentrations of total sugar and free amino acids to those of control seedlings, indicating that 2-ketobutyrate did not induce the phytotoxicity found in chlorsulfuron-treated canola seedlings.

Chlorsulfuron was absorbed slowly but steadily by the treated leaves of seedlings. Twenty-four hours after treatment, 31% of the applied herbicide had been absorbed. However, there was but little transport of chlorsulfuron out of the treated leaf. Only 1.6% of the applied chlorsulfuron was transported out of the treated leaf 24 h after treatment.

Chlorsulfuron treatment reduced assimilate translocation in canola seedlings. This was due to a herbicide effect in two different plant parts, the treated leaf and actively growing plant parts. Chlorsulfuron applied to a fully expanded leaf of a canola seedling reduced assimilate transport out of the treated leaf. Supporting evidence for this was obtained from two different experiments using ^{14}C-sucrose translocation and sucrose exudation. When ^{14}C-sucrose was applied to chlorsulfuron-treated leaves of seedlings, the treated leaves exported less ^{14}C 12 h after herbicide application. Twenty-four hours after treatment, chlorsulfuron-treated leaves had exported 10% of total recovered radioactivity, while control leaves had exported 23%. These results were visualized on autoradiograms. Following ^{14}C-sucrose application, the images of chlorsulfuron-treated leaves were all dark, while those of control leaves were dark only on the ^{14}C-treated spots. Chlorsulfuron-treated excised leaves exuded much less sucrose than control leaves 12 h after herbicide application. These results suggest that the reduction in assimilate translocation can be accounted for by a herbicide effect in the treated leaves. In addition to a herbicide effect in the treated leaves, chlorsulfuron that was translocated from the treated leaves to actively growing plant parts also contributed to the reduction in assimilate translocation. Two lines of supporting evidence were obtained. Firstly, when ^{14}C-sucrose was applied to a leaf adjacent to a chlorsulfuron-treated leaf, that leaf ceased to export ^{14}C 6 h after herbicide application. Secondly, chlorsulfuron applied directly to an unexpanded leaf of a seedling with one expanded leaf and one unexpanded leaf reduced ^{14}C-sucrose transport out of the expanded leaf by decreasing sink strength (sink size and sink activity). The dry weight and protoplast numbers of chlorsulfuron-treated leaves were 40% and 82% lower, respectively, than those of control leaves. In addition, data from sucrose uptake by leaf discs indicated that chlorsulfuron-treated leaves took up less ^{14}C-sucrose than control leaves. These results support the conclusion that the reduction in assimilate transport out of chlorsulfuron-treated leaves was due, in part, to a herbicide effect in actively growing parts of the seedlings. On the basis of these results, the hypothesis that reduction in assimilate translocation in chlorsulfuron-treated susceptible plants is due both to a herbicide effect on reduction in source ability for assimilate export and to a reduction in sink demand for assimilate import, is acceptable.

Although chlorsulfuron-treated leaves exported less sucrose, the treated leaves did not show inhibition of CO_2 fixation or carbohydrate production during the first 24 h. Photosynthesis-related parameters such as chlorophyll content, net carbon exchange rate, and transpiration rate in chlorsulfuron-treated leaves were the same as in control leaves. In addition, concentrations of glucose, fructose, sucrose and starch, and the activity of sucrose phosphate synthase in the treated leaves were similar to those in control leaves. At twenty-four hours after chlorsulfuron treatment, treated leaves respired at a level comparable to control leaves. However, during the 8 hours of the experiment, the respiration rate of treated leaves 24 h after chlorsulfuron treatment was higher than that of control leaves. In addition, the treated leaves showed noticeable changes during that period in nitrogen metabolism, *i.e.*, increases in free amino acids and nitrate reductase activity, suggesting that the reduction of assimilate transport might be linked to these changes. In chlorsulfuron-treated leaves, activities of phosphoenolpyruvate carboxylase and sucrose phosphate synthase, enzymes that control carbon flow between sucrose and amino acids, did not change while activities of nitrate reductase were almost 2-fold higher than those in control leaves. On a time scale, the reduction in sucrose export (12 h) occurred much earlier than the change in nitrate reductase activity (21 h), suggesting that sucrose that was not exported induced the nitrate reductase. With the increased nitrate reductase activity, the accumulation of amino acids in chlorsulfuron-treated leaves could be accounted for, in part, by *de novo* synthesis. Due to lack of conclusive data in SDS-PAGE, it is not known whether the accumulation of amino acids was due to proteolysis or to the impaired utilization of amino acids to protein in chlorsulfuron-treated leaves which showed no herbicide effect on protein content.

Forty-eight hours after chlorsulfuron treatment, treated leaves of canola seedlings showed marked changes in carbohydrate and free amino acid contents. The treated leaves had higher glucose and total sugar contents than control leaves, due possibly to a reduction in sucrose transport. The carbohydrate status in treated (source) leaves affected carbohydrate status in actively growing parts (sinks) of seedlings. The reduction of sucrose transport from chlorsulfuron-treated source leaves could possibly result in a shortage of carbohydrate in sink parts. Carbohydrate starvation is known to induce a number of metabolic and physiological changes. Therefore, in conjuction with herbicidal effects of chlorsulfuron on branched-chain amino acids and pantothenic acid, changes in metabolic and physiological changes by carbohydrate starvation reduce sink growth and eventually lead to death of chlorsulfuron-treated plants.

Chlorsulfuron-treated excised leaves exposed to high CO_2 levels showed an increased net carbon exchange rate. In such leaves, the starch content increased by 24% over that at ambient CO_2 while sugar content was similar to that at ambient CO_2. Exposure of chlorsulfuron-treated leaves to high CO_2 did not overcome the reduction in sucrose transport. These results suggest that an alteration of carbon allocation at high CO_2 prevented an increase in sucrose exudation out of chlorsulfuron-treated leaves.

Overall results from this study show a model for a mechanism of action of chlorsulfuron in the reduction of sucrose translocation in treated susceptible plants. The model is diagrammed in Figure 36. Chlorsulfuron applied to a susceptible plant shows its herbicide effects in two different plant parts, *i. e.*, both in fully expanded (source) leaves and in unexpanded actively growing plant parts (sinks). In both plant parts, chlorsulfuron binds to acetolactate synthase in branched-chain amino acid biosynthesis. As a consequence, synthesis of branched-chain amino acids and pantothenic acid is inhibited. Metabolic processes that require a continuous supply of branched-chain amino acids and pantothenic acid, therefore, are inhibited. However, chlorsulfuron-induced accumulation of 2-ketobutyrate does not inhibit those metabolic processes.

In source leaves of chlorsulfuron-treated susceptible plants, one of the metabolic processes requiring branched-chain amino acids and pantothenic acid is the synthesis of proteins that are involved in sucrose loading into the phloem. Malfunctions of such proteins result in a reduction of sucrose export out of source leaves of chlorsulfuron-treated plants. During that herbicide action, source leaves of treated plants do not lose their photosynthesizing ability. Therefore, source leaves of treated plants produce and allocate carbohydrates at a level comparable to the same leaves of control plants, while they export less sucrose. Sucrose that is not exported is stored as hexoses and, therefore, source leaves of treated plants have a large carbohydrate pool.

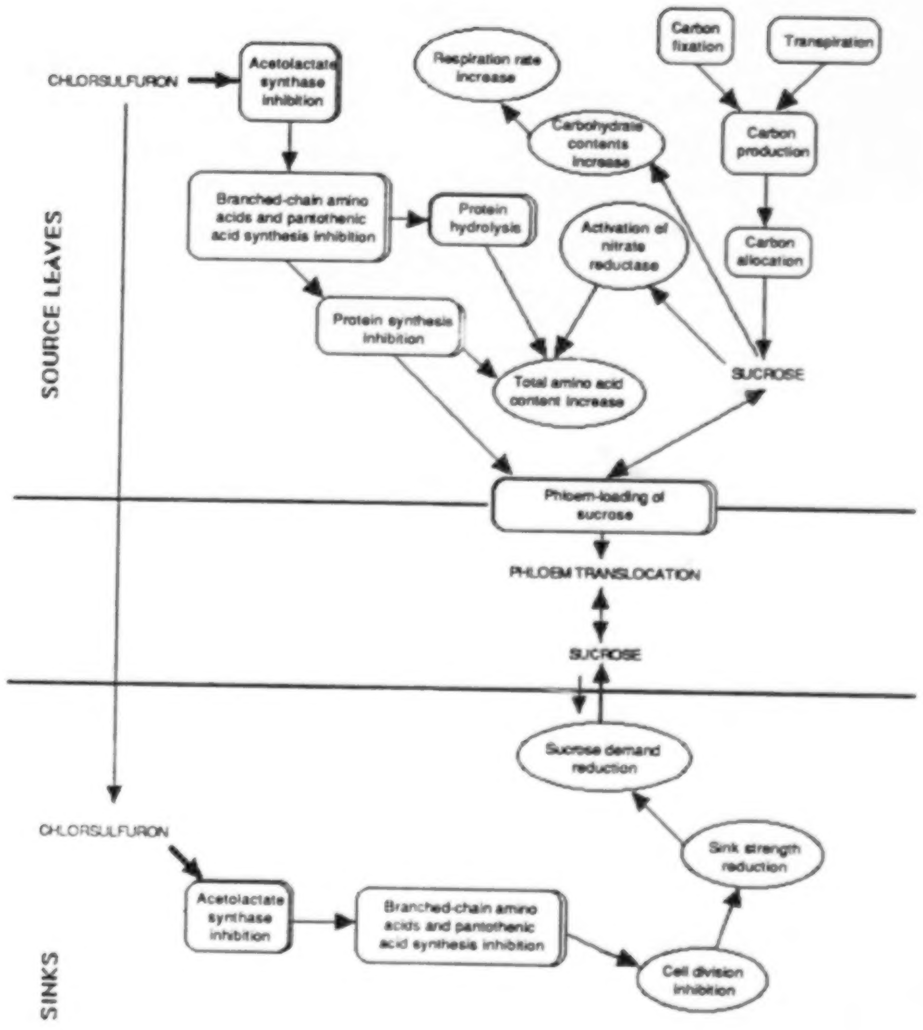
SOURCE LEAVES
CHLORSULFURON
Acetolactate synthase inhibition
Respiration rate increase
Carbon fixation
Transpiration
Carbohydrate contents increase
Carbon production
Branched-chain amino acids and pantothenic acid synthesis inhibition
Protein hydrolysis
Activation of nitrate reductase
Carbon allocation
Protein synthesis inhibition
Total amino acid content increase
SUCROSE
Phloem-loading of sucrose
PHLOEM TRANSLOCATION
SUCROSE
Sucrose demand reduction
CHLORSULFURON
Sink strength reduction
Acetolactate synthase inhibition
Branched-chain amino acids and pantothenic acid synthesis inhibition
Cell division inhibition
SINKS

Figure 36. A schematic diagram of a putative mechanism for a herbicide-induced reduction of sucrose translocation in chlorsulfuron-treated plants. In this figure, a susceptible plant is simplified with a source leaf and a sink. Rectangular boxes indicate unaffected biochemical and physiological processes in chlorsulfuron-treated plants; double boxes represent metabolic processes that are changed; oval boxes indicate final results of changed biochemical and physiological processes that are confirmed in this research.

In sink tissues of chlorsulfuron-treated plants, an interaction of chlorsulfuron with acetolactate synthase inhibits metabolic processes that are related to cell division. The inhibition of cell growth in sink tissues reduces sink strength, which, in turn, reduces the demand for carbohydrates, a source of energy and structural building blocks in many metabolic and physiological processes. A reduction in carbohydrate demand results in a reduction in the phloem loading of sucrose. The model shows that herbicidal effects of chlorsulfuron in sinks and in source leaves of treated plants coordinately reduce sucrose translocation, suggesting a disruption of source-sink relations. This model explains, in part, how chlorsulfuron inhibits growth of susceptible plants.

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APPENDICES

Appendix 1. Composition of a modified Hoagland's nutrient solution. Canola seedlings were grown in the nutrient solution without any visible stress symptoms for 20 days after transplanting.

Constituents	Concentration[1] (µg/l)
NO_3	310
Cl	351
Ca	200
Mg	49
K	235
PO_4	31
SO_4	64
Mn	0.5
B	0.5
Zn	0.05
Cu	0.05
Mo	0.01
Fe[2]	5

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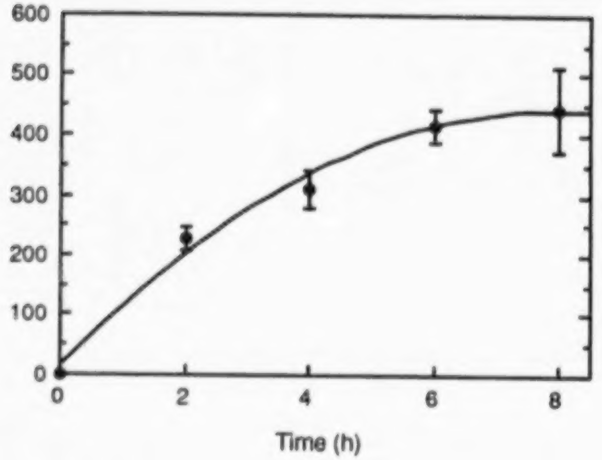
[1] This was made by dissolving 5 ml 1 M $CaCl_2$, 2 ml 1 M $MgSO_4$, 5 ml 1 M KNO_3, 1 ml Fe-EDTA, 1 ml micronutrients containing 1 M H_3BO_3, 1 M $MnCl_2$ $4H_2O$, 1 M $ZnSO_4$ $7H_2O$, 1 M $CuSO_4$ $5H_2O$, and 1 M H_2MoO_4 H_2O in 1 liter.

[2] Fe-EDTA was prepared according to Stegner (1971). EDTA 26.1 g was dissolved in 286 ml of 1M KOH and then 24.9 g of $FeSO_4$ $7H_2O$ and diluted to 1 liter. This solution was aerated for 24 h to produce the Fe-EDTA complex. One ml of this solution provided 5 µg of iron per liter.



Appendix 2. Movement of ^{14}C from the buffer solution to the excised leaves of canola seedlings through transpiration stream following 834 Bq ^{14}C-sucrose application.The first leaves of canola seedlings at the four leaf stage were excised and incubated in potassium phosphate buffer with 1 mM EDTA in a plexiglass chamber for 8 h (section 3.5.1). After oxidation of the excised leaves, ^{14}C activity was measured by a liquid scintillation spectrophotometer.

Appendix 3. Absorption and translocation of ^{14}C in canola seedlings following ^{14}C-chlorsulfuron application. Seedlings were grown as described in section 3.1.1. ^{14}C- and technical grade chlorsulfuron, 1 µg in total volume of 10 µl, were applied to the first leaves of canola seedlings at the four-leaf stage. Total applied ^{14}C was 166 Bq per seedling. A procedure for determination of absorption and translocation of ^{14}C-chlorsulfuron was the same as ^{14}C-sucrose, which was described in sections 3.4.2 and 3.4.4. Means and standard errors (in parenthesis) are based on data from 5 replicates.

Time (h)	Absorption	Translocation	Radioactivity found in: Treated leaf[1]	Adjacent leaf[2]	Apex[3]	Roots	Remain[4]
	(% of total applied ^{14}C activity)						
1	26.0 (4.0)	2.2 (0.8)	7.5 (0.8)	1.0 (0.4)	0.7 (0.3)	0.5 (0.1)	0.7 (0.3)
3	23.8 (1.1)	1.6 (0.1)	8.5 (1.2)	0.6 (0.1)	0.2 (0.1)	0.6 (0.2)	0.4 (0.0)
6	34.7 (6.7)	1.3 (0.2)	13.6 (4.1)	0.5 (0.1)	0.7 (0.2)	0.3 (0.0)	0.5 (0.1)
12	28.9 (2.0)	1.4 (0.3)	11.3 (2.2)	0.5 (0.0)	0.3 (0.0)	0.4 (0.0)	0.6 (0.0)
24	31.5 (1.0)	1.6 (0.1)	13.5 (1.0)	0.6 (0.1)	0.4 (0.1)	0.5 (0.1)	0.5 (0.0)
48	38.4 (1.6)	1.6 (0.1)	13.7 (0.6)	0.9 (0.0)	0.4 (0.1)	0.3 (0.1)	0.4 (0.0)
72	40.2 (2.1)	2.3 (0.1)	16.7 (2.2)	1.2 (0.1)	0.6 (0.3)	0.3 (0.1)	0.8 (0.0)
96	54.2 (1.3)	3.1 (0.3)	17.5 (2.7)	1.9 (0.2)	1.0 (0.0)	0.6 (0.1)	0.7 (0.1)

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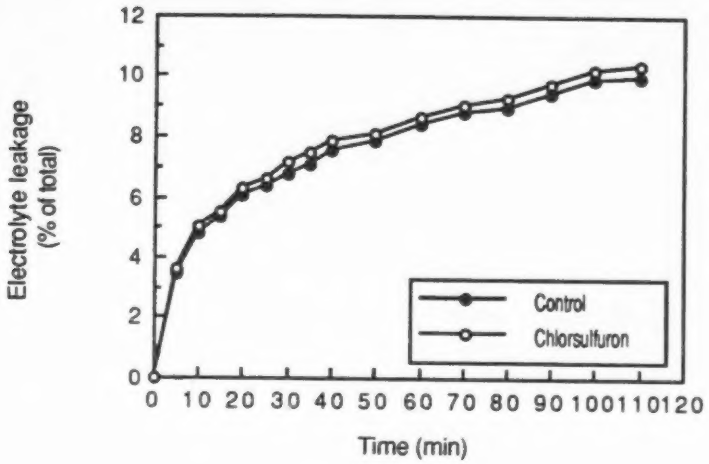
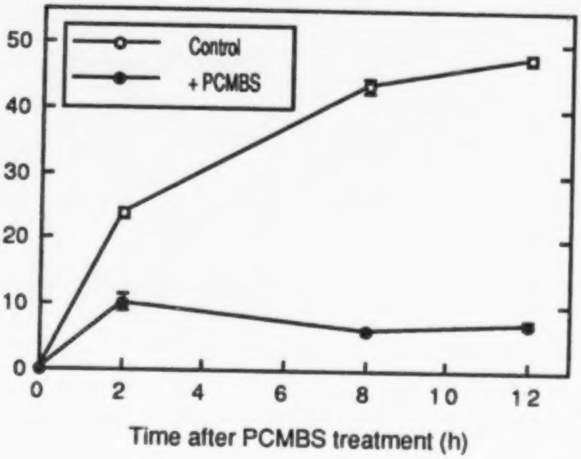
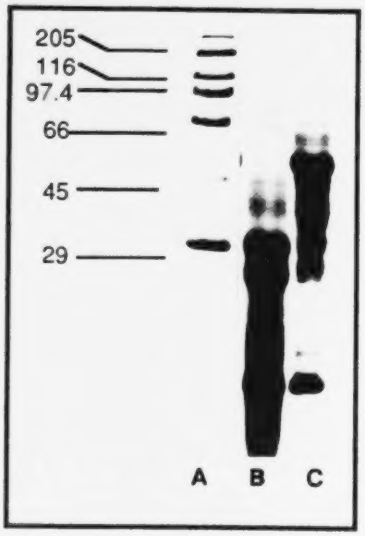
[1] The first leaves of seedlings
[2] The second leaves of seedlings
[3] The third plus the fourth leaves of seedlings
[4] Stem and two cotyledonary leaves of seedlings



Appendix 4. Electrolyte leakage from leaf discs of chlorsulfuron-treated canola leaves. The first leaves of canola seedlings were treated with chlorsulfuron. Twenty-four hours after treatment, leaf discs (7 mm) were made using a cork borer and were incubated in double-distilled-and-deionized water. Conductivity of supernatant was measured every 5 or 10 min. The leaf discs were boiled to determine total conductivity. The conductivity of supernatant was expressed as % of total conductivity.



Appendix 5. Effect of *p*-chloromercuribenzene sulfonic acid on sucrose exudation out of the excised leaves of canola seedlings. The first leaves of canola seedlings at the four-leaf stage were treated with 9.4 kBq ^{14}C-sucrose. Thirty minutes after ^{14}C-sucrose treatment, the treated leaves were excised and cut tips of petioles were placed in potassium phosphate buffer solution with and without 1 mM PCMBS. Two hours after pulse treatment, the excised leaves were placed again in fresh buffer solution which was changed every 2 hours during 8 h. Means and standard errors are based on data from 3 replicates. The results show that the excised leaves of canola seedlings ceased ^{14}C exudation after PCMBS treatment.



Appendix 6. Profiles of total soluble proteins extracted from the third leaf of modified canola seedlings. The leaves were treated with chlorsulfuron forty-eight hours after modification and harvested twenty-four hours after treatment. Proteins were extracted according to Mohapatra (1987), separated by SDS-PAGE, and stained with Coomassie Brilliant Blue. A, molecular markers (kDa); B, control leaf; C, chlorsulfuron-treated leaf.
NOTE: The first leaves of modified seedlings showed numerous protein bands between 45 and 66 kDa (data not shown), whereas the third leaves, as illustrated here, did not show such protein bands.